ALPHA-ADRENERGIC RECEPTOR STIMULATED PHOSPHATIDYLINOSITOL HYDROLYSIS IN THE RAT BRAIN

By

ROBERT EMIL RAULLI

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Chairman: Fulton T. Crews
Major Department: Pharmacology and Therapeutics

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Both phenylethylamines and imidazolines were found to compete for the alpha$_1$ antagonist [^{3}H]-prazosin. Both classes of compounds exhibit two sites of interaction with membrane [^{3}H]-prazosin binding sites when the assay is performed in TRIS buffer. Substituting a

modified Krebs buffer for TRIS buffer shifts the phenylethylamine competition to a one-site interaction, while the imidazoline interaction remained two-site. All phenylethylamines tested stimulate PI hydrolysis in a dose-dependent manner. The imidazolines were not as potent or efficacious in stimulating PI hydrolysis, nor did they exhibit dose-effect relationships. The correlation coefficients between K_d values (from membrane competition for [^{3}H]-prazosin) and ED_{50} values (from PI hydrolysis) were 0.96 and 0.33 for phenylethylamines and imidazolines respectively.

The alpha$_1$ antagonist prazosin was able to inhibit the PI hydrolysis response to phenylethylamines, but not imidazolines. This and the above data suggest that imidazolines are not acting via the classical alpha$_1$ receptor. Imidazolines, in fact, demonstrate dose-dependent inhibition of norepinephrine stimulated PI hydrolysis, thus acting as alpha$_1$ antagonists.

To determine the regulation of norepinephrine-stimulated PI hydrolysis rats were treated with reserpine acutely or chronically (5 mg/kg/day for 4 days or 0.25 mg/kg/day for 14 days). Reserpine, a catecholamine depletor, increases the density of adrenergic receptors in the CNS. Neither reserpine regimen had an effect on the ED_{50} value or magnitude of norepinephrine-stimulated PI hydrolysis. Nor was a change seen in the rate or amount of [^{3}H]-inositol incorporation. Thus, it appears that reserpine, at doses known to increase beta-adrenergic receptor responsiveness, does not increase the norepinephrine-stimulated PI hydrolysis response in rat brain.

This dissertation is dedicated to my parents, Ernest and Jean, the best parents in the world.

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PREFACE

This dissertation is composed of an introduction, four chapters written in standard manuscript style, and a general conclusions section. I apologize for any redundancies that may exist.

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CHAPTER 1
INTRODUCTION

The miracle of the human body is in many ways related to the interaction of the numerous varied cell types and their ability to maintain homeostasis. The division, growth, differentiation, and specialized functions of cells and tissues are carefully regulated by neurotransmitter, hormonal and humoral signals. Most cells are linked to the appropriate signals through receptors on their cell surface. Both the nervous system and immune system communicate with each other, their cellular components and other tissues primarily through these receptors on the exterior of the cell membrane. Although many of these receptors are proteinaceous, they reside in a membrane which is usually half lipid. This lipid plays a major part in the transduction of the signal across the membrane to the interior of the cell. It allows movement of the signal carrying receptors and proteins and serves as a reservoir of messenger molecules which are released from the membrane. Studies in recent years have clearly indicated that there are two key transduction mechanisms involved in autonomic receptors. The beta adrenergic receptors in the heart, lung, liver and other tissues are linked to an enzyme on the inside of the plasma membrane (adenylate cyclase) through a guanine nucleotide coupling protein (Ns). The other two autonomic receptors, i.e. the alpha adrenergic receptor and the muscarinic cholinergic receptor, are linked to the hydrolysis of phosphoinositides, actual lipid components

of the membrane. Both of these signal transduction mechanisms are dependent upon and/or regulated by membrane lipids. In addition, the methylation of phospholipids is regulated by receptors and appears to modulate and/or mediate certain cellular signals in various cells. A fourth type of lipid involvement in signal transduction involves the release of arachidonic acid, the precursor of prostaglandins, leukotrienes and other bioactive metabolites which serve to transmit and/or modulate cellular responses.

Tissues and organs are continuously regulated by signals from the extracellular environment. The nervous, endocrine, and immune systems constantly signal a variety of tissues and cells and simultaneously process signals from those cells resulting in coordination of cellular function. All cells that process extracellular signals need mechanisms to transport that message into the cell. The transduction system usually starts with membrane receptor recognition of the hormone or neurotransmitter (cytosolic receptors for steroidal hormones are notable exceptions). This recognition produces conformational changes in the receptor. Molecular events involved in receptor conformation changes trigger mechanisms by which the signal can be passed from the cell surface through the lipid bilayer to the cytosol. Systems using cyclic AMP as a second messenger are perhaps the best characterized. In the transduction phase, receptor occupation causes the GTP-dependent release of N protein components that migrate through the membrane bilayer. These proteins bind to adenylate cyclase on the inner membrane and stimulate (or inhibit) the production of cAMP. The cAMP produced serves as the second messenger

to stimulate cAMP-dependent protein kinase (for review of this system see Helmreich and Pfeuffer, 1985).

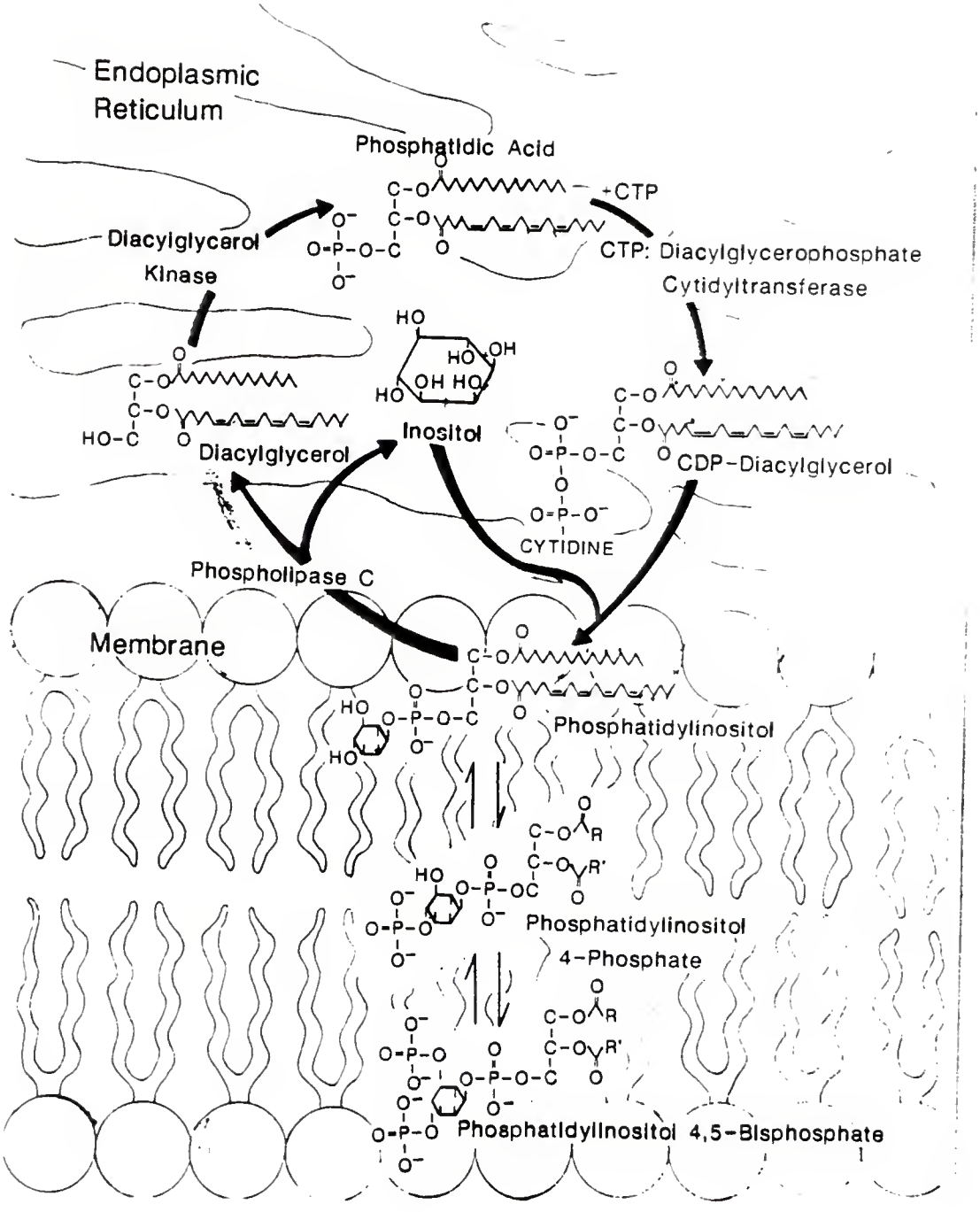
In this introduction, we will discuss the hydrolysis of phosphatidylinositol as a transduction system since lipids are the central component of this signal.

Synthesis of Phosphatidylinositol and Polyphosphatidylinositol

Phosphatidylinositol (PI) is synthesized by the multi-step pathway shown in Figure I-I. Phosphatidic acid (PA) is transformed to CDP-diacylglycerol by the enzyme CTP:diacyl-glycerophosphate cytidyltransferase. CDP-diacylglycerol combines with inositol to form PI and CMP. These processes take place in the endoplasmic reticulum. PI can be further phosphorylated to phosphatidylinositol 4-phosphate (PtdIns 4P) and phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5P2) by phosphatidylinositol kinases. Phosphomonoesterases are also present that convert PtdIns 4,5P2 to PtdIns 4P and PtdIns 4P back to PI. Phosphorylation and dephosphorylation of the inositol head group of PI are believed to take place in the plasma membrane (Hawthorne and White, 1975). PI, PtdIns 4P, and PtdIns 4,5P2 exist in the membrane in "steady-state" concentrations with PI serving as a reservoir for polyphosphoinositides that are hydrolyzed during receptor activation.

Receptor occupation by an appropriate agonist leads to the rapid activation of the enzyme phospholipase-C (PL-C) to yield the hydrolysis products inositolpolyphosphate and diacylglycerol (DAG).

Figure 1-1. The synthetic pathway of phosphatidylinositol. Phosphatidylinositol is synthesized from phosphatidic acid in the endoplasmic reticulum via the above multi-step pathway. In the membrane phosphatidylinositol can be phosphorylated by phosphatidylinositol kinase(s) to phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate.


Endoplasmic
Reticulum
Phosphatidic Acid
+CTP
Diacylglycerol
Kinase
CTP: Diacylglycerophosphate
Cytidyltransferase
Inositol
Diacylglycerol
CDP-Diacylglycerol
CYTIDINE
Phospholipase C
Membrane
Phosphatidylinositol
Phosphatidylinositol
4-Phosphate
Phosphatidylinositol 4,5-Bisphosphate

Until recently the molecular events involved in PL-C stimulation were unknown. Evidence is rapidly accumulating which suggests that PL-C activation may be triggered by a guanine nucleotide binding protein (Npi). It has been found that guanine nucleotides and guanine nucleotide analogs can induce the breakdown of inositol phospholipids in permeabilized cells (Cockcroft and Gomperts, 1985) and in membranes (Litosch et al., 1985; Gonzales and Crews, 1985b). The time course and products produced mimic receptor agonist induced breakdown. From these studies and others we can propose a simplified mechanism. Receptor activation causes the physical uncoupling of a guanine nucleotide binding protein from the receptor. The protein is then free to migrate through the bilayer or, perhaps to the cytosol to interact with PL-C. This interaction causes a stimulation of PL-C which acts to hydrolyze PI.

The PL-C activated by receptor stimulation is believed to be a polyphosphatidylinositol specific one (Abdel-Latif et al., 1977; Berridge, 1983). Abdel-Latif et al. demonstrated a significant loss of ^{32}P from triphosphoinositide with the addition of ACh and eserine to ^{32}P labelled rabbit iris muscle. ACh also decreased the incorporation of ^{32}P into PtdIns 4,5P2, presumably by stimulating breakdown. This decrease in ^{32}P incorporation was accompanied by a large increase in PA and PI labelling with no increase in PA and PI content in the tissue. Berridge and co-workers demonstrated an agonist-dependent increase in inositol 1,4-bisphosphate (DPI) and inositol 1,4,5-trisphosphate (TPI) in blowfly salivary gland, rat parotid gland and rat brain cortex (Berridge et al., 1983).

Streb et al. (1985) report an agonist-induced increase in the levels of TPI of 106% over controls while the levels of DPI were increased by 64% over controls. Time course studies indicate that agonist-induced TPI formation precedes that of inositol phosphate in blowfly salivary gland and GH3 pituitary cells (Berridge et al., 1984; Rebecchi and Gershengorn, 1983; Martin, 1983; Drummond et al., 1984). Together, these results suggest that receptor-activated PL-C exhibits a substrate specificity for PtdIns 4,5P2. Many studies of this type show an increase in inositol 1-phosphate (IP) as well as the increase in inositol 1,4-bisphosphate (DPI). This may be the result of enzymatic cleavage of phosphate groups from TPI. Another interpretation is that PL-C can hydrolyze all phosphatidylinositols and the IP and DPI produced either a) have effects that are specific and as yet unidentified, or b) have no effects but allow for the stoichiometric production of DAG, which can go on to stimulate protein kinase-C. In any case, the hydrolysis of phosphoinositides can produce 2 second messengers (inositolpolyphosphates and DAG) which can initiate cascades involving Ca^{++} mobilization and protein kinase-C activation.

Mobilization of Intracellular Calcium

In the early 1950s, Hokin and Hokin (1953) observed that cholinergic drugs could stimulate the incorporation of ^{32}P into PI and PA of pancreatic slices. Since that time many other hormonal and neurotransmitters have been found to produce similar effects on PI metabolism in a variety of tissues (Berridge and Irvine, 1984). In 1975, Michell made the observation that all tissues that respond to an

agonist with a mobilization of intracellular Ca^{++} also exhibit PI breakdown. He hypothesized that PI breakdown is the precursor of Ca^{++} mobilization (Michell, 1975).

The role of Ca^{++} in this cascade was controversial. Although it was agreed that PI hydrolysis and increased intracellular Ca^{++} were correlated, no cause and effect relationship existed. There were two possibilities: one was that PI hydrolysis was stimulated by a receptor mediated rise in intracellular Ca^{++}; the other was PI hydrolysis was the precursor of Ca^{++} elevation. In support of the second theory, the addition of Ca^{++} ionophore in the presence of high extracellular Ca^{++} failed to stimulate the breakdown of PI in a variety of tissues. However, platelets, polymorphonuclear leukocytes, nervous tissue and others do demonstrate PI hydrolysis with ionophore (Michell and Kirk, 1981). Evidence exists in the platelet that ionophore-stimulated PI hydrolysis has a different mechanism than that of thrombin-induced hydrolysis and, therefore, may not be a good model for this particular study (Lapetina et al., 1981). These studies indicate that although the PI response can be independent of Ca^{++} in many tissues, some tissues exhibit a PI response that is secondary to an increase in Ca^{++}. Gonzales and Crews (1985b) have shown that guanine nucleotides and Ca^{++} can increase inositide hydrolysis in isolated membranes in an additive manner. Thus, it is possible that both Ca^{++} activated and/or Ca^{++} independent hydrolysis of PI can occur depending on the cell type.

An interesting early theory suggesting the rise in intracellular Ca^{++} is due to PI hydrolysis was put forth by Salmon and Honeyman

(1980). They believed that the hydrolysis of PI to DAG to PA by an enzymatic phosphatase was the key event in this cascade. PA was known to be a Ca^{++} ionophore in artificial systems (Tyson et al., 1976). Experiments were performed on isolated frog smooth muscles that exhibited contraction when stimulated with carbachol. They found that carbachol produced an increase in PA content of the cells. In addition, they demonstrated that addition of 1 μM PA could mimic the contractions produced by 100 μM carbachol. Thus they hypothesized that PA was acting as a Ca^{++} ionophore, raising the intracellular Ca^{++} concentrations and causing smooth muscle contraction. However, in a time-course study of vasopressin-induced PI hydrolysis in isolated hepatocytes, the formation of PA was slower than Ca^{++} mobilization. Also, the concentration of vasopressin required to produce maximum Ca^{++} mobilization was much less than that required to produce maximum levels of PA (Thomas et al., 1983). It is possible that PA plays a minor role in the mobilization of Ca^{++}, but more recent studies suggest that the most important factor in mobilization of intracellular Ca^{++} is TPI.

As mentioned above, inositol 1,4,5-trisphosphate (TPI) formation precedes that of other phosphoinositols (Berridge et al., 1984; Rebecchi and Gershengorn, 1983; Martin, 1983; Drummond et al., 1984). These studies lead to the hypothesis that TPI could act as a second messenger to stimulate Ca^{++} release from intracellular pools (Berridge et al., 1984; Rebecchi and Gershengorn, 1983). This hypothesis was put to the test by Streb et al. (1983). Using permeabilized rat pancreatic acinar cells which allow phosphoinositols to cross the

plasma membrane they found that Ins 1,4,5P could release intracellular Ca^{++}. The release was concentration dependent, rapid, and specific for Ins 1,4,5P. Using the mitochondrial poisons antimycin A or oligomycin they determined that the Ins 1,4,5P induced Ca^{++} release was not sensitive to mitochondrial poisons, suggesting a non-mitochondrial Ca^{++} storage site. This storage of Ca^{++} was dependent on the presence of ATP (Biden et al., 1984), and cellular subfractionation studies determined that the storage site was the microsomal fraction i.e. endoplasmic reticulum (Prentki et al., 1984; Streb et al., 1984).

Recently, Irvine et al. (1984) discovered that stimulation of rat parotid gland with carbachol produced the polyphosphoinositol isomer inositol 1,3,4-trisphosphate. Subsequent examination showed that inositol 1,4,5-trisphosphate is rapidly phosphorylated by a 3-phosphokinase to inositol 1,3,4,5-tetrakisphosphate (IP4) (Batty et al., 1985). IP4 is a substrate for 5-phosphomonoesterase which produces inositol 1,3,4-trisphosphate. Using sea urchin eggs, Irvine and Moor (1986) found that microinjection of inositol 1,4,5-trisphosphate could fully activate eggs. Microinjections of inositol 2,4,5-trisphosphate, an analog capable of mobilizing intracellular Ca^{++} but not as potent as the 1,4,5 isomer, determined that the 2,4,5 isomer was incapable of fully activating eggs regardless of concentration. Coinjection of the 2,4,5 isomer with IP4 produced activation of eggs. The explanation offered by Irvine is that the 2,4,5 isomer can cause the mobilization of intracellular Ca^{++} stores, but this alone is not enough to fully activate eggs. Ins

1,4,5-trisphosphate, which can mobilize intracellular Ca^{++} and be metabolized to IP4, can fully activate eggs. Thus, Irvine suggests that IP4 activates an extra source of Ca^{++}, presumably a Ca^{++} channel that allows extracellular Ca^{++} into the cytosol to trigger complete egg activation.

The Role of Protein Kinase C

Since its discovery in 1978 by Nishizuka and coworkers (Takai et al., 1977), protein kinase C (PK-C) has been shown to be very important in regulation of cellular function (Nishizuka, 1984a). It is widely distributed phylogenetically and is found in high concentrations in the mammalian brain and spleen (Kuo et al., 1980), suggesting an important role in nervous and immune system function. The activity of PK-C is dependent on Ca^{++} and acidic phospholipids, particularly phosphatidylserine (PS). Other phospholipids can substitute for PS in vitro, but only in the presence of abnormally high Ca^{++} concentrations (Takai et al., 1979), suggesting that PS is critical for in vivo activity. In vitro, the K_d for Ca^{++} is about 60 μM. In the presence of DAG the K_d for Ca^{++} drops to the low μM range and the affinity for PS but not other membrane lipids is greatly increased (Takai et al., 1979). The tumor-promoting phorbol esters mimic the effects of DAG in vitro. The effects of phorbol esters in vivo are more complex because the stimulation of PK-C is very long-lived as compared to the short-lived stimulation resulting from the very labile DAG. Despite this, there is now little doubt that PK-C is the cellular target for the effects of phorbol esters (for a review of the role of tumor promotion see Nishizuka, 1984b).

As mentioned above, the receptor-stimulated activation of phospholipase C produces two hydrolysis products in the membrane. One is the aforementioned polyphosphatidylinositols, highly polar compounds that migrate to the cytosol where they act as biochemical second messengers. The other product is the highly lipophilic substance diacylglycerol. The lipophilic DAG remains in the lipid bilayer where it forms a quaternary complex with PK-C, membrane PS, and Ca^{++} (Nishizuka, 1984). This active quaternary complex rapidly phosphorylates other cellular enzymes altering their activity. This model is supported by studies that demonstrate a shift of PK-C activity from the cytosol to the membrane when phorbol esters are incubated with parietal yolk sac cells (Kraft and Anderson, 1983). Gonadotropin releasing factor also induces a shift of PK-C activity to the membrane when incubated with isolated pituitary cells, presumably through the receptor-mediated production of DAG (Hirota et al., 1985). It is interesting to note that with normal aging, the activity of PK-C in the cytosol markedly decreases, while the activity associated with the membrane stays the same (Calderini et al., 1987). Since the membrane bound PK-C is likely to be active in vivo, it is not surprising that activity in this fraction is preserved during aging.

Phosphorylation by PK-C has been shown to regulate a wide variety of cellular substrates in vivo and in vitro including EGF receptors (Davis and Czech, 1984), beta-adrenergic receptors (Kelleher et al., 1984), cytoskeletal proteins (Werth et al., 1983), enzymes involved in glucose metabolism (Ahmad et al., 1984), and many others. Like most protein kinases, PK-C has a broad substrate specificity. It will be

difficult to sort out the specific substrates that are most important physiologically. The type of studies most likely to solve this mystery are those combining biochemical evidence of protein phosphorylation by PK-C plus biochemical or physiological changes in response to transmitters or phorbol esters. Despite our ignorance it is quite obvious that PK-C is an important part of the signal transduction system that begins with receptor stimulated hydrolysis of PI.

Combined Effects of Calcium and Protein Kinase C

Receptor-stimulated activation of PL-C can produce a rise in intracellular Ca^{++} and PK-C activity. Thus, two bifurcating "arms" exist in the signal transduction cascade. To examine the physiological effect of an agonist in any system, both arms of the cascade must be considered. In an elegant series of studies in the laboratory of Nishizuka, it was found that one could mimic the effects of thrombin, collagen, and platelet activating factor (PAF) on platelets by adding A23187, a Ca^{++} ionophore, and 1-oleoyl-2-acetylglycerol (OAG), a DAG analog that penetrates the cell membrane to activate PK-C (Kaibuchi et al., 1983; Sano et al., 1983). Addition of both compounds stimulated the phosphorylation of a 20K and 40K molecular weight protein. The degree of phosphorylation of the proteins correlated with the release of serotonin, whose release is also stimulated by thrombin, collagen, and PAF. The addition of OAG only produces phosphorylation of the 40K protein while A23187 only stimulates the phosphorylation of the 20K protein, now known to be myosin light chain kinase, presumably by calmodulin-dependent protein

kinase. In each case, there was no release of serotonin. Serotonin release requires both DAG-PK-C and calcium-activated phosphoprotein. Thus, both pathways together can act synergistically. Synergism of these pathways has also been demonstrated on lysosomal enzyme release in neutrophils (White et al., 1984; Robinson et al., 1984), histamine release from mast cells (Heiman and Crews, 1985), and many other systems (Hirasawa and Nishizuka, 1985).

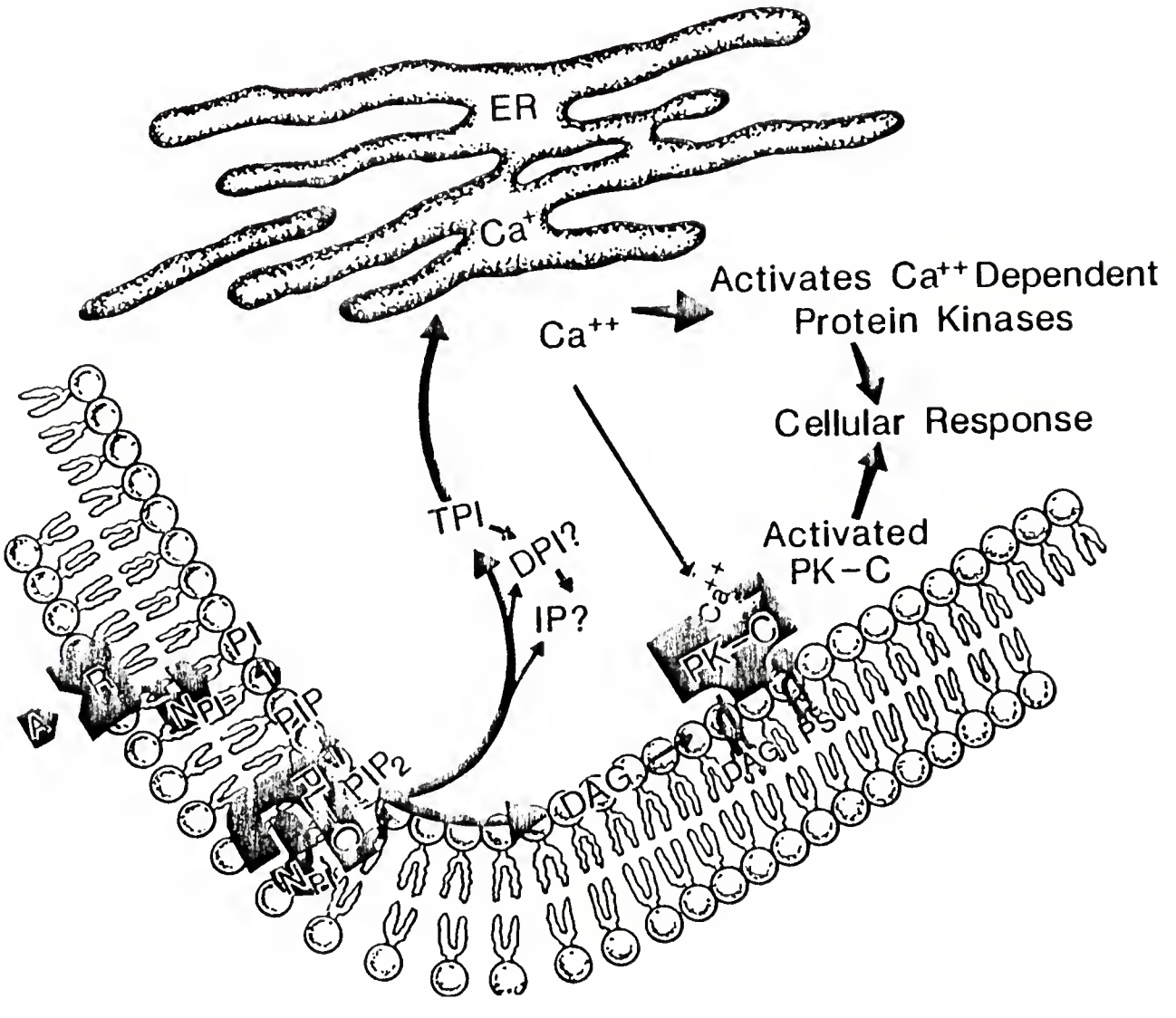
Recent evidence suggests that PK-C exerts a negative feedback on intracellular Ca^{++} levels (Drummond, 1985). In GH3 pituitary tumor cells thyrotropin-releasing hormone (TRH) will stimulate PI hydrolysis to form TPI and DAG. At low TRH receptor occupancy, [Ca]i increases rapidly and declines slowly. As receptor occupancy increases, the Ca^{++} signal duration is decreased due to an inhibitory component. This inhibition is mimicked by phorbol esters and bacterial PK-C. Also the time course of DAG production in these cells agrees with the onset of the inhibitory phase. These data suggest that DAG activation of PK-C may feedback to turn off the increase in the intracellular calcium level.

It is clear that there are tissue differences in the interaction of PK-C stimulation with Ca^{++} mobilization. The differential effect of PK-C on the Ca^{++} signal is probably dependent on the function of that cell and the various mechanisms of regulation of that cell type. In some cases the "arms" of this bifurcating cascade can antagonize each other and strike a balance to regulate homeostasis; in other cases they synergize to perform some cellular function.

Summary of the Phosphatidylinositide Cascade System

Having gone through the various steps of PI signal transduction system, we can now construct a model (Figure I-2). Receptor occupation by an appropriate agonist causes an activation of PL-C, possibly by a guanine nucleotide coupling protein that dissociates from the receptor and binds to PL-C, activating the enzyme. PL-C will hydrolyze PtdIns 4,5P2 to form the products TPI and DAG. The water soluble TPI migrates to the cytosol and somehow effects the release of Ca^{++} from the endoplasmic reticulum. The lipid soluble DAG remains in the membrane to form a quaternary complex with PS, Ca^{++}, and PK-C, resulting in an activation of the kinase. While the effects of TPI on the intracellular Ca^{++} levels are fairly well characterized, the effects of PK-C activation with respect to endogenous substrates and biochemical responses are still unknown.

Figure 1-2. Model of the phosphatidylinositol cascade. Agonist (A) combines with receptor (R) causing the displacement of guanine nucleotide binding protein (Npi). Npi binds to the enzyme phospholipase C (PL-C) which activates the enzyme to hydrolyze phosphatidylinositols of the inner membrane to diacylglycerol (DAG) and the respective inositol phosphate. Inositoltrisphosphate (TPI) diffuses to the endoplasmic reticulum where it causes the release of sequestered Ca^{++}. DAG forms a quaternary complex with protein kinase C (PK-C), phosphatidylserine (PS), and Ca^{++}. This complex is the activated form of PK-C. The PK-C and the released Ca^{++} can then act to regulate cellular processes leading to the response.


ER
Ca+
Ca++
Activates Ca++ Dependent
Protein Kinases
Cellular Response
TPI
DPI?
IP?
Activated
PK-C
PI
PIP
PIP2
DAG
PS
PK-C

CHAPTER 2
BRAIN REGIONAL DISTRIBUTION OF ALPHA$_1$ ADRENERGIC-STIMULATED PHOSPHOINOSITIDE HYDROLYSIS

Introduction

The distribution of alpha$_1$ receptors varies regionally within the brain (Bremner and Greengrass, 1979; Young and Kuhar, 1980; Jones et al., 1984). Although the results of these studies vary, probably due to methodological differences, in general the cortex, hippocampus, and olfactory bulb have a high density of alpha$_1$ receptors while the brain stem, hypothalamus, striatum, and cerebellum have moderate to low densities of alpha$_1$ receptors. Neuronal alpha$_1$ receptors are coupled to phosphoinositide (PI) hydrolysis as a second messenger (Brown et al., 1984; Minneman and Johnson, 1984; Schoepp et al., 1984). Thus, we have studied the regional distribution of alpha$_1$ agonist-stimulated PI hydrolysis for norepinephrine (NE) and other alpha$_1$ agonists in the rat brain.

Previous experiments with muscarinic receptors have suggested that efficacy differences between agonists show larger variation than potency (Gonzales and Crews, 1984). In fact, muscarinic agonists have been separated into classes by binding properties and correlation to agonist efficacy (Fisher et al., 1983). Furthermore, the efficacy of muscarinic partial agonists was found to vary among brain regions. Thus, we studied the maximal responses to full and partial alpha$_1$ agonists in several brain regions.

Classically, NE is a full alpha$_1$ agonist. Phenylephrine (PHEN), methoxamine (METH), and dihydroergotamine (DHE) are also characterized as alpha$_1$ agonists. 6-Fluoronorepinephrine (6-FLNE) is a phenylethylamine derivative with a decreased beta-adrenergic component (Auerbach et al., 1981). The imidazoline derivative oxymetazoline (OXY), believed to be mostly alpha$_2$-adrenergic, behaves as an alpha$_1$ agonist in rat vas deferens (Minneman et al., 1983); therefore, it was included in this study.

Methods

Dissection of brain regions. Brains were dissected as follows. The brain was rapidly removed and placed upright on a dissection platform wetted with warm KRB buffer. The olfactory bulbs were removed at the stalk, cut in half longitudinally, and placed on a tissue chopper disk for slicing. The cortices were dissected using a razor blade to cut slices 1 to 2 mm in thickness, avoiding underlying white matter. After the cortical slices were removed, the remaining cortex and corpus callosum were peeled back to reveal the intact hippocampus. The septal hippocampal connections were cut and both hippocampi were removed intact. The brain was then rotated so that the inferior surface was up. A frontal section cut was made at the optic chiasm. The striatum was dissected from the frontal piece by trimming away the surrounding regions. The striatum was sliced with a razor blade into slices 1 to 2 mm thick and then further sliced with a tissue chopper. The hypothalamus was dissected by making frontal section cuts at the optic chiasm and just caudal to the mammillary bodies. Sagittal cuts were made approximately 2 mm lateral to the

central plane. A coronal cut was then made through the anterior commissure to denote the upper surface of the hypothalamus. The brain stem was sliced into sheets with a razor blade and further sliced with a tissue chopper.

Determination of phosphoinositide hydrolysis. Phosphatidyl-inositol hydrolysis was performed as described by Gonzales and Crews (1984). Brain slices were carefully sliced and placed in warm, oxygenated Krebs-Ringer bicarbonate buffer (KRB buffer). Slices were minced with a McIlwain tissue chopper at 350 μm in perpendicular directions. Slices were transferred to 50 ml conical flasks and dispersed. The slices were washed four times with fresh oxygenated KRB buffer. [^{3}H]-inositol (myo-[2-^{3}H]inositol, 16.3 Ci/mmole, Amersham, UK) was added to a final concentration of 0.1 to 0.3 μM in a volume approximately four times that of the settled slices and incubated for 1 hour at 37°C with enough agitation to prevent the slices from settling. At the end of this incorporation period the slices were washed with fresh, oxygenated KRB buffer, allowed to settle and brought to a volume approximately 4 times that of the packed slices. While gently swirling the slices 50 μl were transferred to polypropylene tubes containing 190 μl of KRB buffer with 10 mM LiCl substituted for NaCl. Reactions were started with the addition of 10 μl of appropriate concentrations of agonist or buffer. The tubes were gassed with O_2:CO_2 (95:5), capped tightly, and shaken in an incubator at 37°C. The reaction was stopped with the addition of 1 ml chloroform/methanol (1:2, v/v). An additional 0.35 ml of distilled water and 0.35 ml of chloroform was added, and the tubes

were tightly capped, shaken, and centrifuged to separate the phases. An 0.75 ml aliquot of the aqueous layer was removed, diluted to 3 ml with distilled water, and a 1 ml aliquot of Dowex-1 (50% v/v) slurry was added to each tube. The slurry containing bound inositol phosphates was poured into a polypropylene column with a fritted disk. Inositol phosphates were eluted directly into scintillation vials with 5 ml of 0.1 M formic acid/1.0 M ammonium formate and 10 ml Liquiscint added. An aliquot of the chloroform layer was placed into glass scintillation vials, evaporated under an air stream, and 3 ml of OCS scintillation fluid was added. The samples were counted in a Beckman LS7500 scintillation counter at a 37% efficiency. The data were expressed as total DPM of [^{3}H]-inositol phosphates released/total DPM [^{3}H]-inositol incorporated x 100, otherwise called fractional release x 100 (FR x 100).

Results

Stimulation of phosphoinositide hydrolysis by alpha-adrenergic agents in various brain regions. Maximum responsiveness for NE was determined by performing dose-response curves in the rat cerebral cortex. Brain region studies were performed with NE concentrations 3 to 10-fold greater than what was needed for maximum response in cerebral cortex. OXY reaches maximum at 1 mM in the rat cerebral cortex; therefore, the data for OXY have been separated from the phenylethylamine data.

NE-stimulated PI hydrolysis showed regional variation. The most robust response to NE was found in the hippocampus, approximately twice as responsive as the brain stem (weakest). The rank order for

regional responsiveness to NE is: hippocampus ≥ olfactory bulb ≥ cortex > striatum ≥ hypothalamus > brain stem (Figures 2-1 and 2-2).

Although OXY is fairly potent at stimulating contraction of vas deferens (Minneman et al., 1983), little or no phosphoinositide hydrolysis occurred at concentrations as high as 100 μM. Regional differences were found at OXY concentrations of 1 mM. The cortex and striatum exhibited the greatest response, approximately three times greater than the brain stem. The rank order of responsiveness for 1 mM OXY is: cortex = striatum ≥ hippocampus >> hypothalamus > brain stem.

Examination of the relative efficacies of other phenylethylamine compounds (Table 2-1) shows that the efficacy remains fairly consistent across brain regions. METH is more effective than PHEN in brain stem and olfactory bulb, while PHEN is more effective in striatum. While these differences show a trend towards significance, we conclude no great differences in efficacy occur from region to region.

The relative efficacies for OXY are shown in Table 2-2. Pronounced differences in regional relative efficacy exist for the maximal OXY concentration. Clearly, the regional rank order of figure efficacy for OXY contrasts with all the phenylethylamines studied.

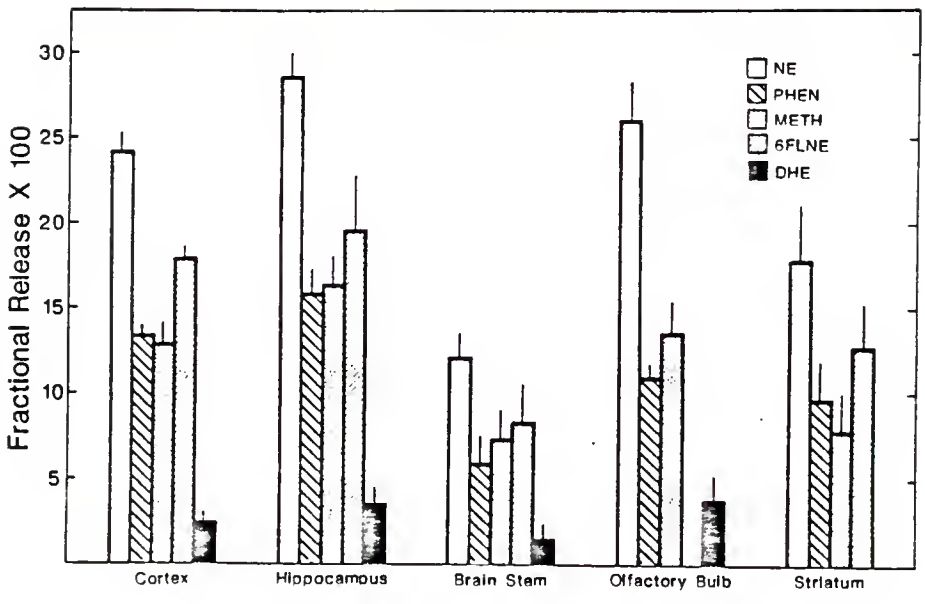


Figure 2-1. Fractional release of various alpha-adrenergic agonists in rat brain regions. Each bar represents 6 determinations from 2 separate experiments. Error bars indicate S.E.M. NE, norepinephrine; PHEN, phenylephrine; METH, methoxamine; 6FLNE, 6-fluoronorepinephrine; DHE, dihydroergotamine.

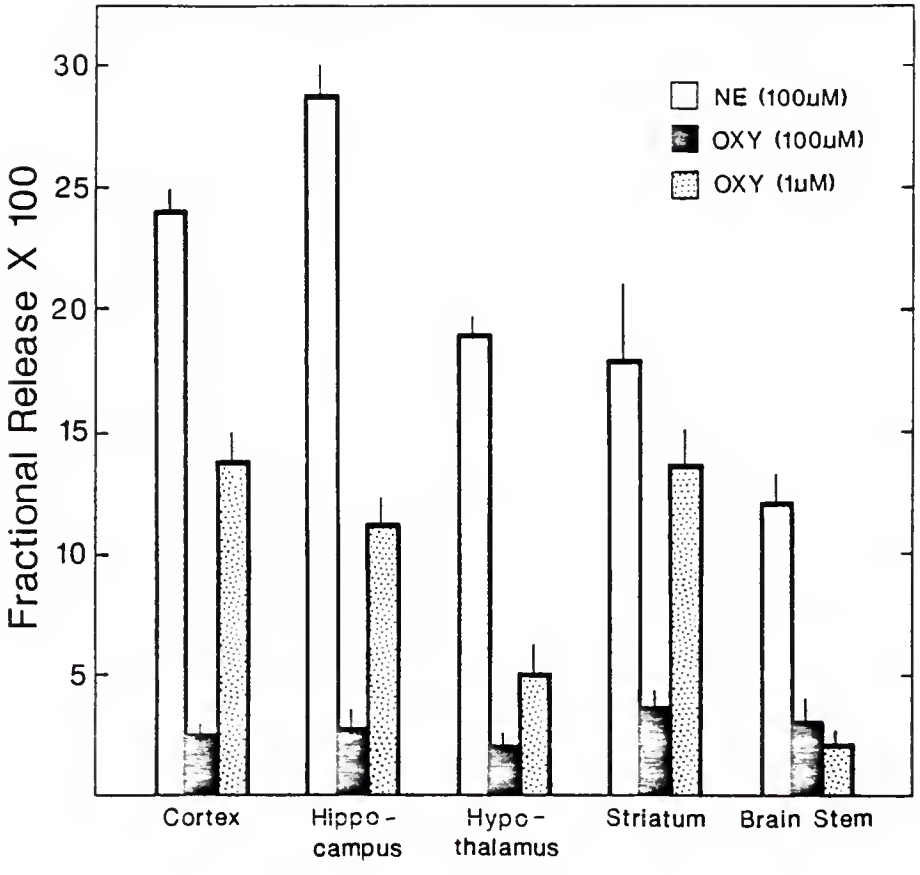


Figure 2-2. Comparison of the fractional release values from 100 μM norepinephrine and concentrations of oxymetazoline (OXY) in various rat brain regions. Each bar represents nine determinations from 3 separate experiments.

TABLE 2-1

RELATIVE EFFICACIES OF PHENYLETHYLAMINE DERIVATIVES IN VARIOUS BRAIN REGIONS

DRUG	CORTEX	HIPPOCAMPUS	BRAIN STEM	OLFACTORY BULB	STRIATUM
NE	1.00	1.00	1.00	1.00	1.00
PHEN	0.55	0.55	0.48	0.43	0.54
METH	0.53	0.57	0.60	0.52	0.43
6FLNE	0.74	0.68	0.68	----	0.72
DHE	0.09	0.12	0.12	0.14	----

Fractional release values were determined in various brain regions (6 determinations from 2 experiments). Relative efficacy values were determined by taking the average fractional release for each drug and dividing by the average fractional release value for NE in that brain region. NE, norepinephrine; PHEN, phenylephrine; METH, methoxamine; 6FLNE, 6-flouronorepinephrine; DHE, dihydroergotamine.

TABLE 2-2

RELATIVE EFFICACY OF OXYMETAZOLINE IN VARIOUS BRAIN REGIONS

OXY	CORTEX	HIPPOCAMPUS	BRAIN STEM	HYPOTHALAMUS	STRIATUM
100μM	0.11±0.05	0.09±0.04	0.26±0.12	0.11±0.04	0.14±0.03
1mM	0.57±0.07	0.39±0.04	0.18±0.07	0.27±0.05	0.76±0.05

Fractional release (FR) values (9 determinations from 3 experiments) were determined and the average FR for 100 μM or 1mM oxymetazoline (OXY) in a brain region was divided by the average FR for NE in that brain region. Data are expressed as relative efficacy ± S.E.M.

Discussion

Previous studies have shown that the number of $alpha_1$ adrenergic receptors varies regionally within the brain. Bremner and Greengrass (1979), using [^{3}H]-prazosin as their label, find the highest density of $alpha_1$ receptors in the frontal cortex > hypothalamic-pre-optic area > striatum > cerebellum > basal hypothalamus. Studies using [^{125}I]-HEAT show the highest density of $alpha_1$ receptors in the frontal cortex > hippocampus > brain stem $\geq$ olfactory bulb > striatum > cerebellum (Jones et al., 1984). We have shown high levels of responsiveness to NE in hippocampus, olfactory bulb, and cerebral cortex, with a moderate response from striatum and hypothalamus. Brain stem was the least responsive region tested. Cerebellum barely exhibits a response to 100 μM NE (Gonzales and Crews, 1985a). These data are in general agreement with radioligand studies performed on rat brain homogenates and autoradiographic analysis of receptor distribution.

All drugs tested are partial agonists compared to NE. The phenylethylamine derivatives retain their relative efficacy coefficients from region to region while OXY, surprisingly, varies greatly. In addition, the PI response to OXY rose sharply from almost zero to maximum with a ten-fold increase in concentration. Ruffolo et al. (1977) found cross desensitization in the rat vas deferens between different imidazolines but not between imidazolines and phenylethylamines. Taken together, these data suggest that OXY may have a different mechanism of PI hydrolysis than phenylethylamines.

Therefore, we decided to compare the interaction of a larger group of imidazolines to phenylethylamines on $alpha_1$ binding sites and alpha-stimulated PI hydrolysis in cerebral cortex.

CHAPTER 3
DIFFERENCES IN IMIDAZOLINE AND PHENYLETHYLAMINE ALPHA-ADRENERGIC AGONISTS: COMPARISON OF BINDING AFFINITY AND PHOSPHOINOSITIDE RESPONSE

Introduction

The neurotransmitter NE can stimulate both $alpha_1$ and $alpha_2$ receptors in the brain $alpha_1$ receptors are coupled to hydrolysis of phosphoinositides (PI) (Kendall et al., 1985; Gonzales and Crews, 1985a) while neuronal $alpha_2$ receptors are negatively coupled to adenylate cyclase (Kitamura et al., 1985). In addition, these receptor responses can be selectively inhibited by prazosin (α_1) or yohimbine (α_2). In rat cerebral cortex, for example, prazosin is three orders of magnitude more potent than yohimbine for inhibition of NE-stimulated PI hydrolysis (Kendall et al., 1985).

Thus, in the brain, α_1 and α_2 responses are dissimilar and distinguishable with selective receptor antagonists.

Such is not the case for vascular smooth muscle. A chemically diverse variety of alpha adrenergic are known to contract vascular smooth muscle. These contractions can be mediated by postsynaptic α_1 and/or α_2 receptors (Timmermans et al., 1979; Constantine et al., 1980; Madjar, 1980). NE can induce contractions via both α_1 and α_2 receptors. Unlike the thousand-fold difference seen in brain and rat aorta, prazosin is only 20 times more potent than rauwolscine in cat mesenteric artery (Skarby et al., 1983), ten times more potent in dog

splenic artery, and three times more potent in dog splenic vein (Hieble and Woodward, 1984). Thus, the distinction between α_1 and α_2 mediated effects are less than clear.

Phenylethylamine derivatives of NE, such as phenylephrine and methoxamine are selectively blocked by prazosin (van Meel et al., 1981). The imidazoline cirazoline (CIR) is also selectively blocked by prazosin (van Meel et al., 1981; Cavero et al., 1982). Other imidazoline compounds, such as oxymetazolines (OXY), tramazoline (TRAM), and naphazoline (NAPH), while believed to act at α_2 receptors (Timmermans and van Zwieten, 1982), may also contract via an $alpha_1$ component. For example, experiments on the $alpha_1$ receptor-induced contraction of rat vas deferens smooth muscle induced by NAPH, TRAM and OXY indicate these compounds have an efficacy of 0.50, 0.68 and 0.72 compared to NE, respectively. In addition, 20 nM prazosin can shift the dose-response curve for contraction 10-20 fold, suggesting an $alpha_1$-receptor mediated contraction (Minneman et al., 1983).

We have compared classical phenylethylamine adrenergic drugs to imidazolines in order to better understand the interaction of these compounds with the $alpha_1$ receptor. Our model system, rat cerebral cortex, contains $alpha_1$ receptors which are coupled to phosphatidylinositol (PI) hydrolysis and selectively inhibited by prazosin (Kendall et al., 1985; Gonzales and Crews, 1985a). We report here that there are clear differences in the binding properties and agonist activity of imidazoline alpha agonists when compared to phenylethylamine alpha agonists.

Methods

Radioligand Binding. Rats were decapitated and their brains placed into either ice-cold 50mM TRIS/HCl buffer containing 1mM EDTA pH adjusted to 7.4 or modified Krebs-Ringer bicarbonate buffer (KRB buffer) (minus glucose). Cerebral cortex was dissected away and weighed. The cortex was minced in 20 volumes per wet weight of ice-cold buffer and homogenized using a Tekmar Tissuemizer at setting 50 for 30 seconds. The homogenate was centrifuged at 42000 x g for 10 min at 4°C. The supernate was discarded and the pellet resuspended in 20 volumes of ice-cold buffer. The membranes were washed two additional times and resuspended in 20 volumes of ice-cold buffer. An aliquot was taken for protein determination. The assay was started with the addition of approximately 0.3 mg of protein into test tubes containing the buffer indicated, 10 nM [^{3}H]prazosin ([7-methoxy-^{3}H]prazosin, 83 Ci/mmole, Amersham, UK), and the indicated concentration of competing ligand in a total volume of 0.5 ml. Non-specific binding was determined in the presence of 10 μM phentolamine. Non-specific binding was approximately 20% of total binding. Incubations were performed at 25°C for 30 min and terminated by rapid filtration onto Whatman GF/C filter paper. Filters were washed three times with ice-cold buffer. Filters were placed in scintillation vials and 10 ml of Liquiscint added. Vials were shaken for 1 hour and counted in a Beckman LS7500 scintillation counter at 37 % efficiency.

Phosphatidylinositol Hydrolysis. See "Methods" section, Chapter 2.

Data Analysis. Competition curves were analyzed using the iterative, non-linear curve fitting program of Fletcher and Shrager as described by McKinney and Coyle (1982). The criterion for determination of one vs. two site fit was that previously used by Fisher et al. (1983) and is as follows: when comparing the sum of squared residuals for one vs. two site fit, if one was at least five fold smaller than the other, the smaller sum of squared residuals was considered the best fit. For cases where the sum of squared residuals was less than five fold different, t-tests between actual occupancy and predicted occupancy for one- and two-site fits were examined. If the data were significantly different from one of the best fit models, that model was eliminated and the other accepted. If both or neither models were significantly different from the actual data, t-tests between the predicted one and two site models were examined; if the two models were not significantly different from each other the best fit was taken to be one site.

ED_{50} and IC_{50} values were determined using probit analysis as outlined by Goldstein (1964).

Protein Determination. Protein concentrations were determined using the Coomassie blue dye method (Bradford, 1976).

Drugs. The source of drugs used is as follows: (-)-norepinephrine bitartrate, (-)-epinephrine bitartrate, l-phenylephrine HCl, oxymetazoline HCl, and naphazoline HCl (Sigma Chemical Co., St. Louis, MO), tramazoline HCl (Dr. Karl Thomae GMBH Biberach, FRG), cirazoline HCL (L.E.R.S. Synthelabo Paris, France), prazosin HCl (Pfizer Inc. Groton, CT), phentolamine mesylate (Ciba-Geigy Corp.

Summit, NJ), alphamethylnorepineprine HCl (Sterling-Winthrop Rensselaer, NY), methoxamine HCl (Burroughs Wellcome Co. Research Triangle Park, NC), guanfacine (gift from Dr. Micheal Katovich).

Results

Agonist competition curves for [^{3}H]-prazosin binding in cerebral cortical membranes. To determine the apparent affinity of the various alpha agonists studied for alpha$_1$ receptors, competition curves were performed. To investigate the actions of sodium and other ions as well as consistency in binding and response experiments, binding was done in both TRIS buffer and KRB buffer. All agents tested inhibit [^{3}H]-prazosin binding (Figures 3-1 and 3-2). In TRIS buffer, all agonists were found to best-fit two sites of interaction (Figure 3-1 and Table 3-1). Comparison of the high affinity K_d values indicate that the imidazolines phentolamine and oxymetazoline had the highest affinity, in the low nmol range, followed by EPI $\geq$ METH > TRAM $\geq$ NAPH $\geq$ CIR $\geq$ NE with K_d values around 1 μM (Table 3-1). The least potent agents were αMENE and phenylephrine. Low affinity sites show a marked difference between the classes of compounds. Phenylethylamines have low affinity sites with K_d values greater than 100 μM, whereas the low affinity sites for imidazolines have K_d values less than 30 μM. The potency series for low affinity sites suggests that the imidazolines have a higher affinity and therefore might be several times more potent than phenylethylamines. Thus, the potency series for low affinity sites is quite different from that of high affinity sites. Furthermore, imidazolines interact with alpha$_1$ receptors with an affinity equal to or greater than that of NE.

To examine the effects of sodium and other ions competition curves were also performed in KRB buffer. The imidazolines again have higher affinities than NE. The imidazolines CIR (Figure 3-2) and TRAM (data not shown) do not completely displace all the [^{3}H]-prazosin sites, leaving about 20% of the total label at saturating concentrations of competitor. OXY and PHENTOL have the highest affinities with K_d values in the low nM range; CIR, EPI, TRAM, PHEN, and NE all have K_d values around 1 μM, whereas METH and αMENE have the lowest affinity with K_d values in the mid-μM range. Examination of best-fit analysis shows that phenylethylamines exhibit a one-site best-fit in KRB buffer while the imidazolines exhibit a two-site interaction (Table 3-2). It is clear that imidazolines in general have higher affinities for [^{3}H]-prazosin sites in both TRIS buffer and KRB buffer.

Agonist-stimulated PI hydrolysis dose-response curves. The high affinity for alpha$_1$ receptors exhibited by imidazolines and their known ability to contract vascular smooth muscle led us to test their ability to stimulate phosphoinositide hydrolysis. To determine the the potency and efficacy of various agonists, dose-response curves were performed (Figures 3-3 and 3-4). The phenylethylamines, EPI, NE, and αMENE are full agonists (Figure 3-3). Phenylephrine and methoxamine (not shown) are partial agonists. The order of potency is EPI > NE $\geq$ PHEN > αMENE > METH. Characteristic of alpha receptor responses, stimulated PI hydrolysis is stereospecific as (+)-NE is less potent and, surprisingly, less efficacious than (-)-NE (Table 3-3). The alpha$_2$ agonist clonidine (100 μM) did not stimulate PI

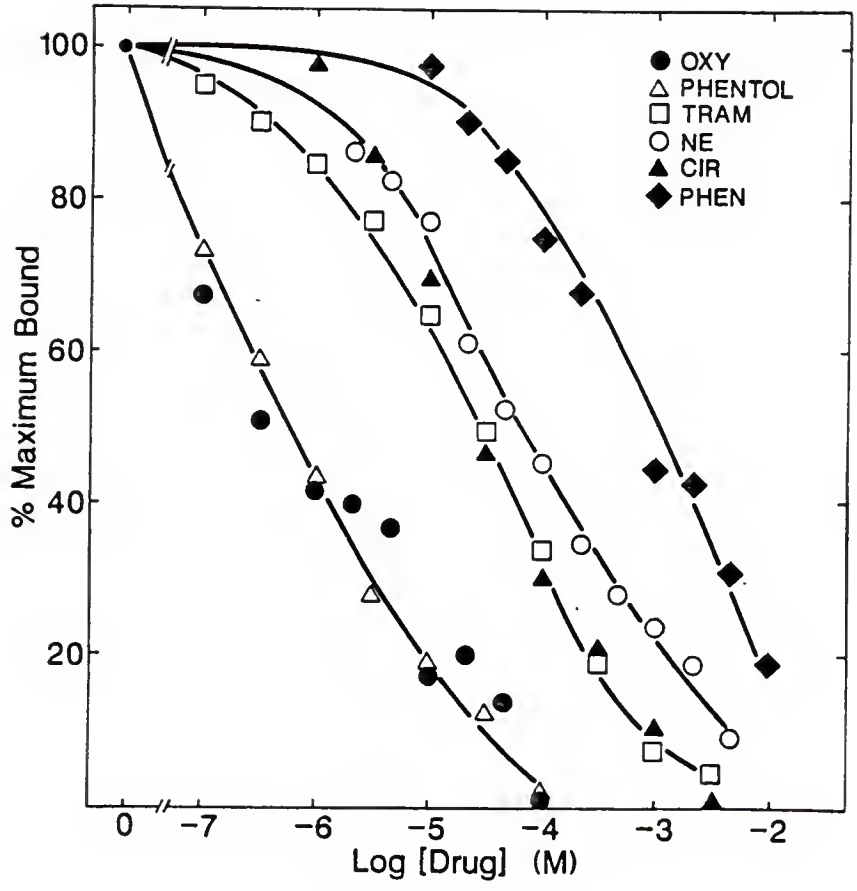


Figure 3-1. Competition curves for [^{3}H]-prazosin binding by various alpha-adrenergic compounds in TRIS buffer. Assays were performed on rat cerebral cortex as described in "Methods." Values are expressed as percent of maximum specific binding. For the sake of clarity only the data points for oxymetazoline and cirazoline are presented. For norepinephrine and oxymetazoline, data points represent 9 determinations from 3 separate experiments. Other points represent 6 determinations from 2 separate experiments.

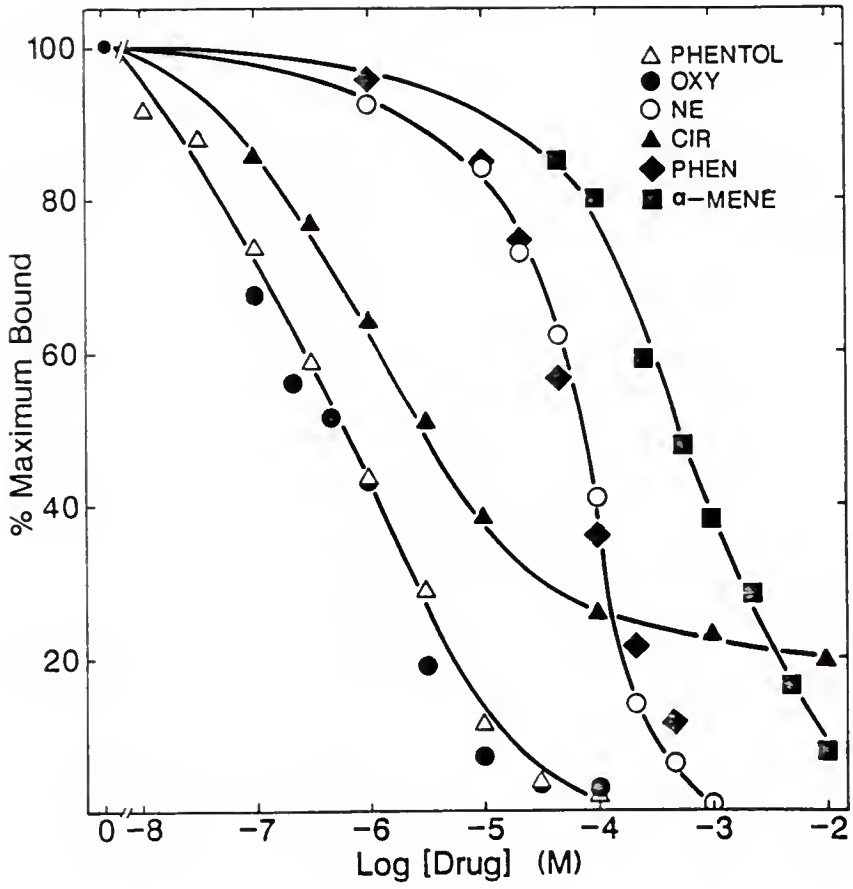


Figure 3-2. Competition curves for [^{3}H]-prazosin binding by various alpha-adrenergic compounds in modified KRB buffer. Data are presented as in Figure 3-1. Data points for NE represent 9 determinations from 3 separate experiments. All other data points represent 2 separate experiments performed in triplicate.

TABLE 3-1

ALPHA-ADRENERGIC COMPETITION FOR [^{3}H]-PRAZOSIN BINDING SITES IN TRIS BUFFER

	BEST FIT	N	K_d (μM) HIGH	K_d LOW
Norepinephrine	2 Site	3/3	1.44	298
Epinephrine	2 Site	2/2	0.44	271
Phenylephrine	2 Site	2/2	11.75	540
α Methylnore-pinephrine	2 Site	2/2	5.20	249
Methoxamine	2 Site	3/3	0.87	112
Oxymetazoline	2 Site	3/3	0.024	2.87
Cirazoline	2 Site	2/2	1.34	21.43
Tramazoline	2 Site	2/2	1.11	17.31
Naphazoline	2 Site	2/2	1.20	27.55
Phentolamine	2 Site	2/2	0.01	3.29

Computer generated kinetic binding parameters obtained from ligand competition for [^{3}H]-prazosin binding in 50mM TRIS buffer, pH 7.4. Competition assays and data analysis were performed as described in "Methods."

TABLE 3-2

ALPHA-ADRENERGIC COMPETITION FOR [^{3}H]-PRAZOSIN BINDING SITES IN KREBS-RINGER BUFFER

	BEST FIT	N	K_d (μM) HIGH	K_d LOW
Norepinephrine	1 Site	3/3	3.20 ± 0.22	---
Epinephrine	1 Site	2/2	0.84	---
Phenylephrine	1 Site	2/2	3.15	---
∝ Methylnore-pinephrine	1 Site	2/2	58.20	---
Methoxamine	1 Site	3/3	36.9 ± 13.0	---
Oxymetazoline	2 Site	2/2	0.0026	0.12
Cirazoline	2 Site	2/2	0.10	335.0
Tramazoline	2 Site	2/2	0.80	309.0
Naphazoline	1 or 2	2	0.76/0.03	--/73.0
Phentolamine	2 Site	2/2	0.03	97.0

Computer generated kinetic binding parameters obtained from ligand competition for [^{3}H]-prazosin binding in modified KRB buffer (minus glucose). Competition assays and data analysis were performed as described in "Methods."

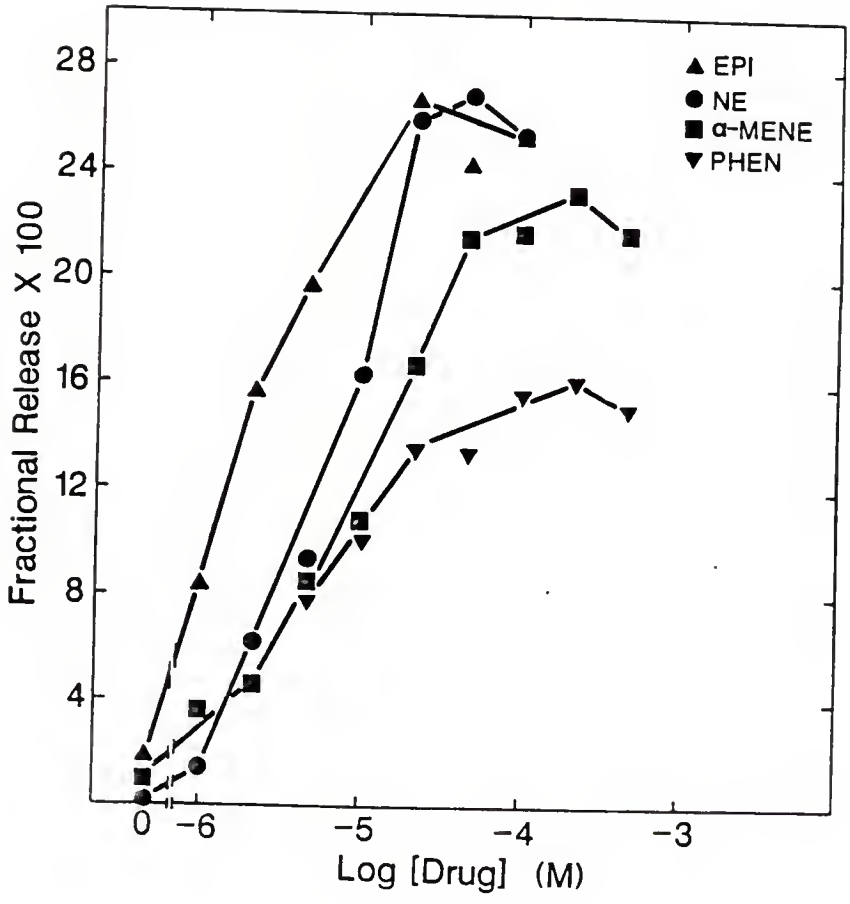


Figure 3-3. Dose-response curves for phenylethylamine stimulation of phosphoinositide hydrolysis in rat cerebral cortex. Data are expressed as Fractional Release x 100 (see "Methods," Chapter 2). Data points represent at least 9 determinations from at least 3 separate experiments. Error bars were omitted for clarity, but at no point exceeded 2 fractional release units.

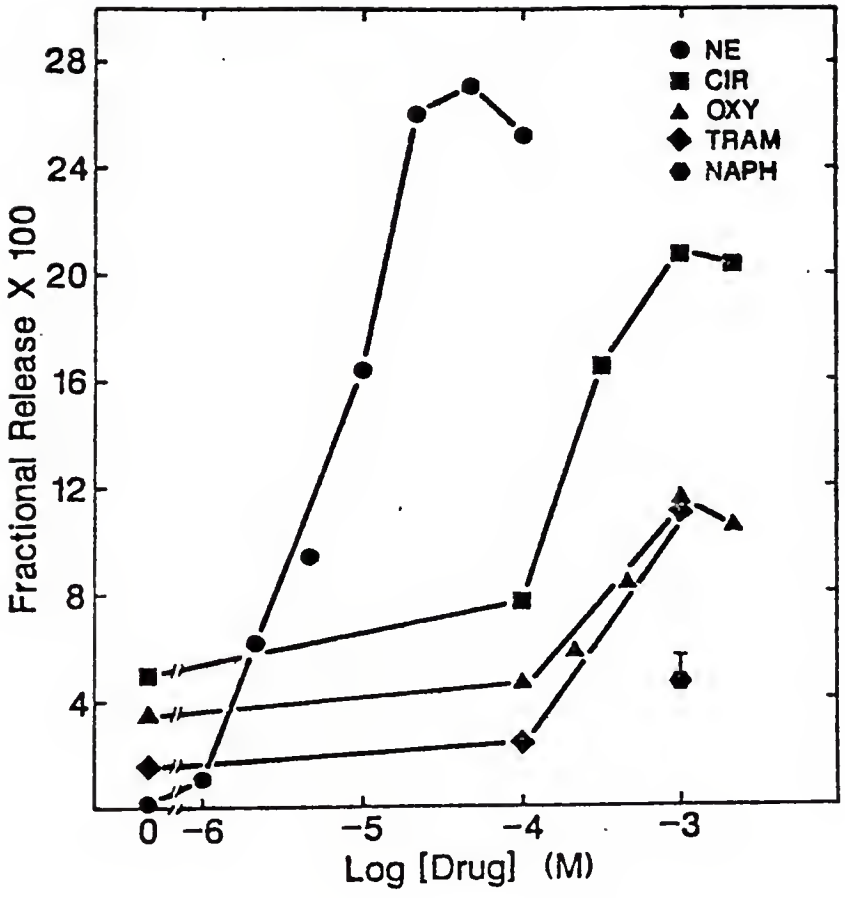


Figure 3-4. Dose-response curves for imidazoline stimulation of phosphoinositide hydrolysis in rat cerebral cortex. Data are expressed as described in Fig. 3-3. Data points represent at least 6 determinations from at least 2 separate experiments. The dose-response curve for norepinephrine is presented for the sake of comparison. The single point plus S.E.M. bar representing naphazoline is the maximum response as determined from three dose-response curves.

TABLE 3-3

ED_{50} AND EFFICACY VALUES FOR VARIOUS ALPHA AGONISTS

Drug	Concentration	ED_{50} (μM)	Rel. Efficacy
EPI	100	1.41 ± 0.10	1.00
NE	100	3.29 ± 0.54	1.00
PHEN	100	5.06 ± 0.98	0.66
∝MENE	100	19.08 ± 2.66	0.95
METH	100	37.57 ± 3.62	0.64
(+)NE	1000	110.76 ± 29.00	0.50
CIR	1000	250.00*	0.76
OXY	1000	500.00*	0.46
TRAM	1000	600.00*	0.42
NAPH	1000	600.00*	0.15
DHE	100	----	0.09
6-FLNE	100	----	0.81
CLON	100	----	0.00
GUANF	1000	----	0.80

ED_{50} values are determined as described in "Methods." Relative efficacies are calculated from the maximal response to agonist divided by the maximal response to norepinephrine.
* Due to the uncharacteristic shape of the imidazoline dose-response curves, the normal probit method of ED_{50} determination was abandoned and the ED_{50}s were approximated by eye.

hydrolysis. The guanidine derivative guanfacine, which acts via the $alpha_2$ receptor, is efficacious but not potent with respect to PI hydrolysis.

The dose-response curves for the imidazoline class of compounds is shown in Figure 3-4. All imidazolines tested exhibit little or no response until concentrations of about 100 μM are reached. Imidazolines have a sharp dose-response curve, from essentially zero to maximum in a ten-fold concentration range. The efficacies range from 0.15 for NAPH to 0.76 for CIR (Table 3-3).

Correlation between K_d values and ED_{50} values for various alpha agonists. Figure 3-5 shows the relationship between K_d values obtained using KRB as the buffer and ED_{50} values for various agonists. The correlation coefficient for the five phenylethylamines is 0.96. Using the high affinity or the low affinity constant from Table 3-2, the imidazoline compounds do not approach the line of unity (r=0.33 and r=0.15 respectively). Correlation of the K_d values for high affinity sites obtained using 50 mM TRIS buffer (pH 7.4) as the buffer to ED_{50} values resulted in a correlation coefficient of 0.23 for the phenylethylamines. Removal of the methoxamine values brings the correlation coefficient to 0.71 for phenylethylamine high affinity sites in TRIS buffer. Again, the imidazolines did not approach the line of unity. These data clearly demonstrate the difference between the two classes of compounds. Phenylethylamines exhibit a one to one response characteristic of tight coupling between receptor occupation and biochemical response. Imidazolines, although quite potent in their ability to bind $alpha_1$ receptors, are weak in eliciting a

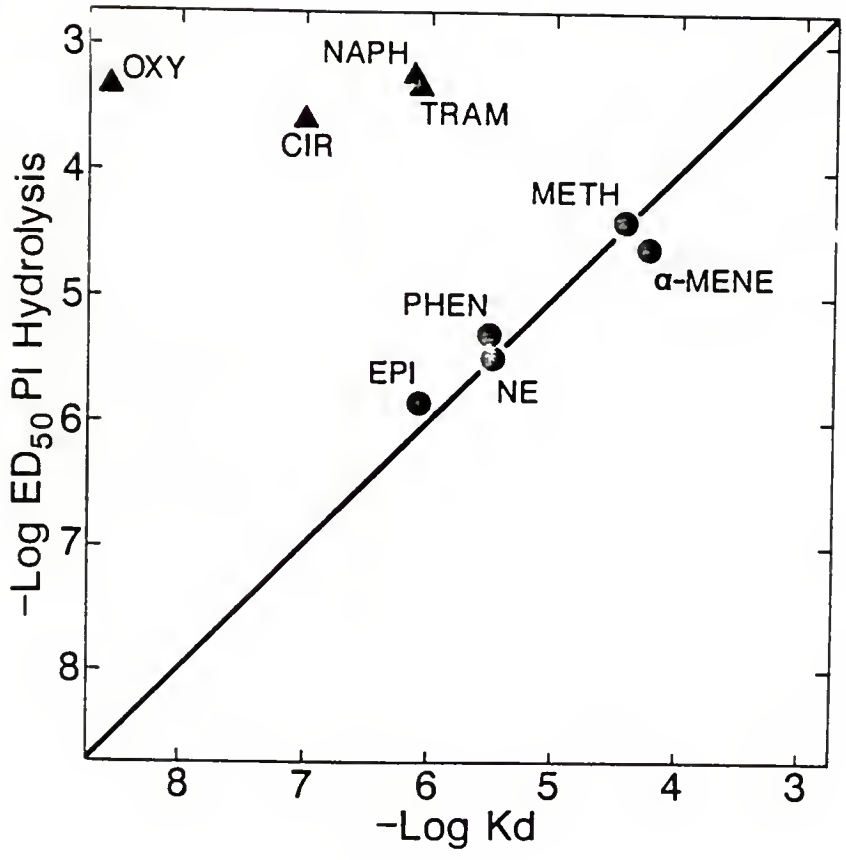


Figure 3-5. Correlation between K_d values determined from competition curves performed in KRB buffer to ED_{50} values for agonist stimulated PI hydrolysis. All experiments were performed in rat cerebral cortical membranes or tissue slices. The data are taken from Tables 3-1 through 3-3. The line represents the line of unity. CIR, cirazoline; EPI, epinephrine; ∝MENE, alphamethylnorepinephrine; METH, methoxamine; NAPH, naphazoline; NE, norepinephrine; OXY, oxymetazoline; PHEN, phenylephrine; TRAM, tramazoline.

biochemical response. The apparent high affinity for alpha$_1$ sites shown by imidazolines contrasts with the weak potency for stimulation of PI hydrolysis.

Antagonism of PI hydrolysis response by prazosin. To determine whether or not these compounds were acting via alpha$_1$ receptors, the ability of prazosin to antagonize the response was tested (Figure 3-6). As expected 1 μM prazosin was able to inhibit the PI response to all phenylethylamines. The imidazoline-stimulated PI response was not inhibited by 1 μM prazosin, nor was the guanfacine-induced PI response inhibited. Similar results were obtained using 10 μM prazosin (data not shown). These data suggest that imidazolines do not stimulate PI hydrolysis via alpha$_1$ receptors.

Ability of imidazolines to antagonize NE-stimulated PI hydrolysis. The potent receptor binding properties of imidazolines combined with their low efficacy suggest these compounds may exhibit antagonist properties. To determine if the imidazolines can antagonize NE-stimulated PI hydrolysis, dose-response curves for oxymetazoline in the presence and absence of 100 μM NE were performed (Figure 3-7). Increasing concentrations of OXY inhibit the NE-stimulated PI hydrolysis. The stimulation by NE is almost completely inhibited at 100 μM OXY. At this point, OXY begins to stimulate PI hydrolysis and the NE + OXY curve exactly follows the OXY curve. Similar results are obtained with CIR, TRAM and NAPH (data not shown). These data indicate that imidazolines can antagonize NE-induced stimulation of PI hydrolysis. It is interesting that the response curve in the presence of 100 μM NE follows the imidazoline

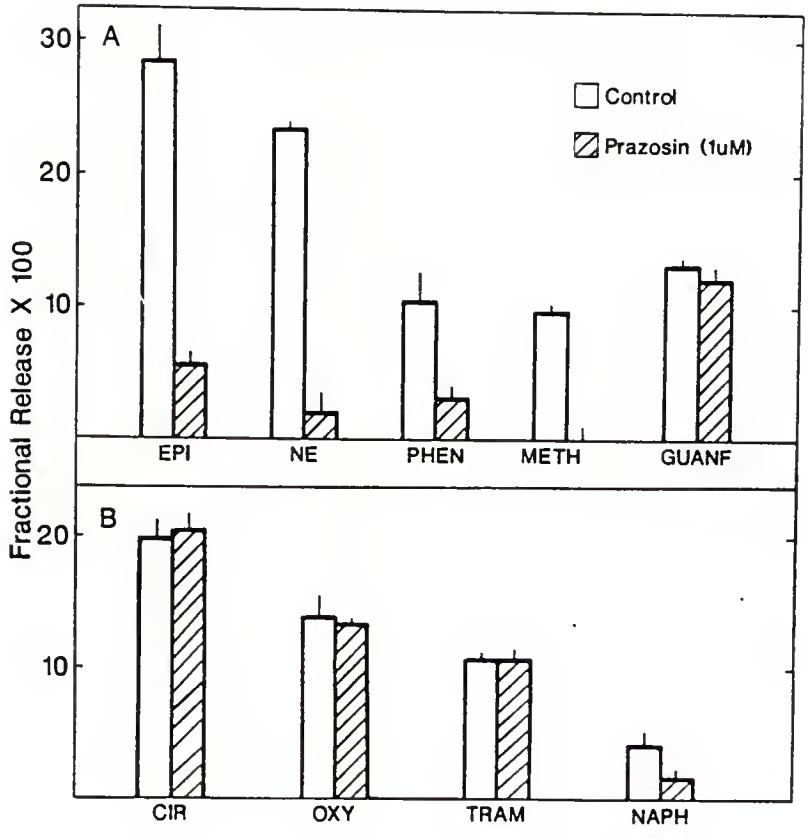


Figure 3-6. Inhibition of agonist-stimulated PI hydrolysis by the alpha$_1$ antagonist prazosin. Each bar represents nine determinations from three separate experiments. Error bars represent the S.E.M. Abbreviations are as in Figure 3-5, with the exception of GUANF, guanfacine.

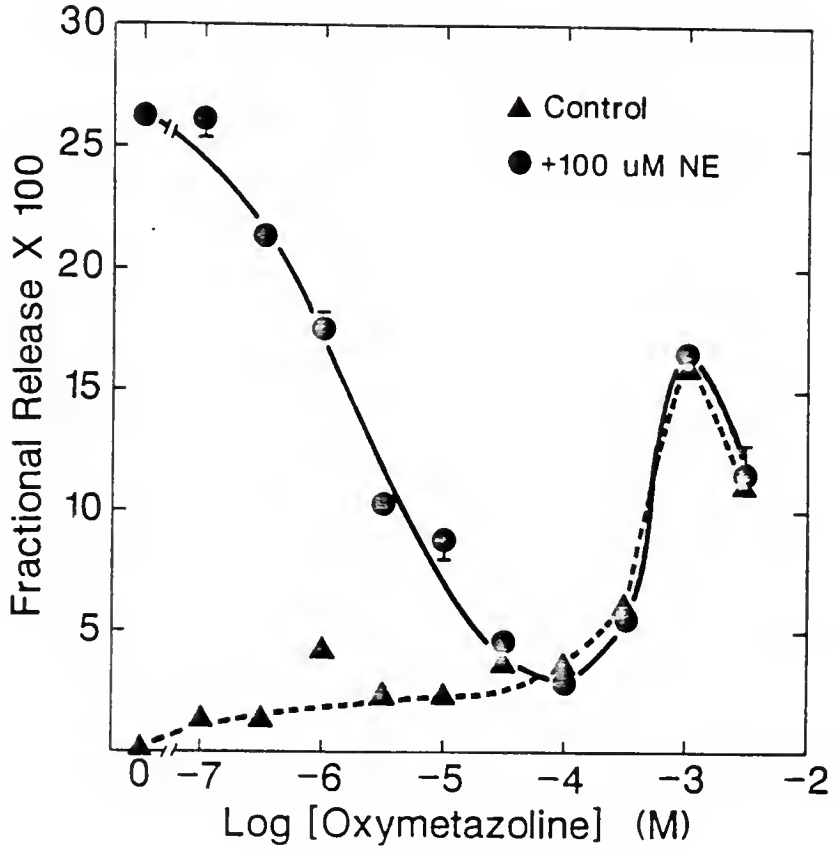


Figure 3-7. Oxymetazoline and oxymetazoline plus norepinephrine stimulation of PI hydrolysis. Data are expressed as FR X 100. Each data point represents nine determinations from three separate experiments. Error bars represent the S.E.M. NE, norepinephrine.

biochemical response. The apparent high affinity for alpha$_1$ sites hypothesis that imidazolines are partial agonists and again suggests a mechanism other than alpha$_1$-receptor stimulated PI hydrolysis.

Discussion

All adrenergic compounds tested compete with [^{3}H]-prazosin for alpha$_1$ receptors. Using 50 mM TRIS buffer, all tested compounds exhibit two sites of interaction. Our data and that of others (Glossmann and Hornung, 1980; Morrow and Creese, 1986) suggest two affinity subtypes for alpha$_1$ agonist binding. In addition, the potency series for our data are in agreement with Hornung et al. (1979). Clear differences arise between phenylethylamines and imidazolines when the competition curves are performed in modified KRB buffer. In this ionic millieu, imidazolines retain two sites of interaction while phenylethylamines are converted to a one-site interaction profile. This conversion of phenylethylamines to a one-site interaction is in agreement with Glossman and Hornung (1980) who, in rat brain membranes, calculates Hill coefficients in the presence and absence of 150 mM NaCl. Sodium shifts the Hill coefficient for NE, EPI, ∝MENE, and PHEN from less than one to one. This shift is particularly dramatic for NE and ∝MENE. The Hill coefficient for NAPH was unaffected by Na^+. Thus, it appears that sodium ion causes the conversion of the phenylethylamine binding profile to one-site.

Dose-response curves for agonist stimulated PI hydrolysis show glaring differences between the two classes of compounds. Phenylethylamines exhibit classical dose-response relationships. The calculated ED_{50} values correlate well with K_d values from competition

curves performed in KRB buffer. In contrast, imidazolines have sharp peaks of activity, starting around 100 μM and reaching maximum at 1 mM. The ED_{50} values for imidazolines do not correlate with any of the high or low K_d values from competition curves performed in either TRIS buffer or KRB buffer. The lack of correlation between ED_{50} values and K_d values for imidazolines suggests a non-alpha$_1$ receptor mediated mechanism of PI hydrolysis. This is also supported by the fact that the alpha$_1$ receptor antagonist prazosin was unable to inhibit the response due to imidazolines. Antagonists for various other receptor systems, including rauwolscine, atropine, haloperidol and others, also were unable to inhibit imidazoline-induced PI hydrolysis (data not shown).

The finding that imidazolines can bind to alpha$_1$ receptors and stimulate PI hydrolysis, along with the fact that imidazolines contract smooth muscle, would suggest imidazolines are alpha$_1$ agonists. Figure 3-6, however, demonstrates that imidazolines act as antagonists at the alpha$_1$ receptor. At 100 μM OXY the PI response to NE is almost completely inhibited. This finding is supported by work on the alpha$_1$ receptor-mediated reduction of [^{3}H]-acetylcholine release from isolated rat atria (McDonough et al., 1986), where it was determined that cirazoline and other imidazolines inhibit the NE-induced reduction of [^{3}H]-acetylcholine release. In addition, OXY and CIR displace the dose-response curve for epinephrine-stimulated PI hydrolysis in hepatocytes (Garcia-Sainz et al., 1985). Our data are further supported by the fact that tolazoline and phentolamine, known alpha$_1$ antagonists, are imidazoline derivatives. Thus, imidazolines

are antagonists at alpha$_1$ receptors that somehow stimulate PI hydrolysis, leading to a cellular response.

Alpha$_1$ receptor activation is associated with PI hydrolysis in vascular smooth muscle cells (Villalobos-Molina et al., 1982; Garcia-Sainz et al., 1985). Ambler et al. (1984) demonstrated a correlation between PI hydrolysis and Ca^{++} mobilization in BC3H-1 muscle cells. Chiu et al. (1987) obtained similar results in isolated rat aorta. It is likely that the alpha$_1$ receptor-stimulated PI hydrolysis provides for the release of intracellular Ca^{++} stores that may act, directly or in combination with other events, to stimulate smooth muscle contraction. However, Chiu and co-workers (1986) find that in rat aorta, NE, PHEN, and CIR stimulate both the influx of extracellular Ca^{++} and release of intracellular Ca^{++} stores, while other agonists stimulate the influx of extracellular Ca^{++} exclusively. Contractions of the rat aorta by OXY, for instance, are completely dependent on extracellular Ca^{++} (Godfraind et al., 1982). Also, although CIR can stimulate contraction via intracellular and extracellular Ca^{++} pathways, pretreatment with phenoxybenzamine renders the pressor response to CIR labile to Ca^{++} channel blockers (Ruffolo et al. 1984). It has been postulated that there is a single alpha$_1$-adrenoceptor able to activate two separate pathways of Ca^{++} mobilization or a single alpha$_1$ receptor that is exclusively coupled to either PL-C or receptor-stimulated Ca^{++} channels (Chiu et al., 1986). Therefore, it possible that contractions elicited by imidazolines are mediated through an alpha$_1$ receptor whose stimulation leads exclusively to external Ca^{++} influx. NE stimulates both this external Ca^{++}-coupled

receptor and a PL-C coupled receptor, while imidazolines block the PL-C-coupled $alpha_1$ receptor. Our data are consistent with the above hypothesis; however, this hypothesis does not explain why imidazolines stimulate PI hydrolysis.

Phospholipase-C is a Ca^{++}-dependent enzyme and its activity is affected by changes in intracellular Ca^{++} concentrations. The Ca^{++} ionophore A23187, presumably via Ca^{++} activation of PL-C, can stimulate PI hydrolysis (Michell and Kirk, 1981; Gonzales et al., 1986). In addition, veratrine, which opens Na^{+} channels, can stimulate PI hydrolysis in rat brain, probably by activation of voltage-dependent Ca^{++} channels (Maier and Rutledge, 1987). Therefore, it is possible imidazoline receptor-activated Ca^{++} flux may be responsible for the observed PI hydrolysis in these experiments.

In summary, the interaction of imidazolines and phenylethylamines at $alpha_1$ receptors, and stimulated PI hydrolysis shows striking differences between the two classes of agonist. Also, imidazolines can act as antagonists to NE-stimulated PI hydrolysis. Imidazoline-stimulated PI hydrolysis is not blocked by a variety of receptor antagonists including the $alpha_1$ receptor antagonist prazosin. It is possible that imidazoline-stimulated PI hydrolysis observed in our experiments is the result of receptor-stimulated Ca^{++} influx.

CHAPTER 4
REGULATION OF RAT BRAIN ALPHA$_1$ RECEPTORS

Introduction

Many neuronal and hormonal signal transduction systems are known to be regulated by agonist stimulation (Fleming et al., 1973). Changes in the normal levels of stimulation will induce changes within the system to maintain homeostasis. Thus, an increase or decrease in synaptic levels of neurotransmitter will generally cause a reciprocal change in the responsiveness of the target tissue.

Reserpine, a catechol depletor, causes postjunctional supersensitivity in the cat nictitating membrane (Trendelenburg, 1966), brain (Ungerstedt et al., 1975), and cardiovascular tissue (Carrier, 1975). In addition, treatment with reserpine will increase beta-adrenergic receptor density (Greenberg and Weiss, 1979) and beta-adrenergic stimulation of adenylate cyclase activity (Dismukes and Daly, 1974; Williams and Pirch, 1974). Alpha$_1$ receptors coupled to PI in the rat brain have not been studied to determine if they follow classical super- and sub-sensitivity as established with smooth muscle preparations and beta-adrenergic coupling to adenylate cyclase. To determine if alpha$_1$ receptor coupled PI hydrolysis exhibits supersensitivity, we studied the PI response to NE in control and reserpine treated rats after acute and chronic reserpine regimens.

Methods

Animals. Male Sprague-Dawley rats (200-300g) were housed in a well ventilated room with food and water available ad libitum. Lights were on between 8:00 a.m. and 4:00 p.m. Drugs were administered by i.p. injection. Animals were killed by decapitation and their brains rapidly removed and placed in ice cold 50 mM TRIS buffer adjusted to pH 7.4 with HCl for preparation of membranes, or 37°C KRB buffer for preparation of brain slices.

Drugs. Reserpine was dissolved in propylene glycol:ethanol: water (6:1:4) vehicle and sonicated 5 to 10 minutes. Reserpine was administered by i.p. injection. Control rats received vehicle in volumes identical to the treated group on a per kg basis.

Phosphoinositide hydrolysis. See "Methods," Chapter 2.

Radioligand binding. See "Methods," Chapter 3.

Adrenergic stimulation of adenylate cyclase in cerebral cortical slices. Adrenergic stimulation of adenylate cyclase in cerebral cortical slices was carried out by incubating the slices with norepinephrine or isoproterenol as modified by Vetulani et al. (1976). Cerebral cortices were dissected as described in Chapter 2. In addition, the dissected cortices were further cut into slices 0.35 mm thick in two perpendicular planes using a McIlwain tissue chopper. Each control or drug-treated group consisted of cerebral cortical slices obtained from three rats. Immediately following their preparation, the slices were transferred to flasks containing 50 ml of physiologic buffer, pH 7.4 (Kakiuchi and Rall, 1968) at 37°C which had been bubbled with O_2/CO_2 (95:5). After the slices were gently

dispersed, they were washed four times with fresh physiologic buffer at 37°C over a period of 10 minutes. The washed slices were then dispersed in 50 ml fresh physiologic buffer and incubated for 45 minutes at 37°C. The slices were continuously aggitated gently and bubbled with a slow stream of O_2/CO_2 during the incubation. After the 45 minute incubation, the slices were allowed to settle and the supernatant buffer was removed and discarded. The slices were resuspended in 50 ml of fresh physiologic buffer containing 5 mM theophylline and were incubated an additional 15 minutes at 37°C with gentle aggitation and continuous bubbling of O_2CO_2. Following the 15 minute incubation, the slices were again allowed to settle and the supernatant buffer discarded. The slices were resuspended in 10 ml of fresh physiologic buffer containing 5 mM theophylline at 37°C. One milliliter aliquots of well-dispersed slices were then transferred to separate tubes containing 2 ml of physiologic buffer with 5 mM theophylline at 37°C for incubation with buffer alone for determination of basal cAMP production or with agonist. Incubation with buffer or agonist was carried out in triplicate. To begin stimulation, 0.33 ml of physiologic buffer or 10-fold concentrated agonist was added. Preliminary experiments indicated a dose-dependent increase in cAMP production stimulated by norepinephrine or isoproterenol, with maximum stimulation occurring with 100 μM of either agonist. Immediately after addition of buffer or agonist, the tubes were rapidly gassed with a high pressure stream of O_2/CO_2, tightly capped, and gently aggitated for 10 minutes at 37°C. Care was taken during this incubation to prevent the slices from settling on

the bottom of the tubes. Time course experiments done previously indicated that maximum stimulation of cAMP production in cerebral cortical slices by either norepinephrine or isoproterenol occurred after 10 minutes of incubation. To stop the stimulation, the slices were allowed to settle, and the supernatant buffer was aspirated and discarded. Preliminary experiments found no detectable cAMP in the supernatant buffer. Each tube containing slices then received 3 ml boiling-hot 1 N HCl, and the slices were immediately homogenized with a Tekmar Tissuemizer at setting 50 for approximately 30 seconds. The homogenates were centrifuged at 27,000 x g for 30 minutes. The pellets were dissolved in 1 ml 0.5 N NaOH for protein determination. Each incubation tube contained from 6.0 to 10.0 mg of protein. The supernatants were evaporated using a Savant rotary evaporator, and the residues remaining were dissolved in 1 ml 25 mM TRIS-HCl buffer, pH 7.0 containing 5 mM theophylline and saved for assay of cAMP.

The cAMP content of the dissolved residue samples was determined in triplicate using a protein binding assay modified from Brown et al. (1971). 50 μl of sample was incubated with 60 μl of 25 mM TRIS-HCl buffer, pH 7.0, 50 μl 0.8 nM [^{3}H]-cAMP (Amersham; 41 Ci/mmole), and 40 μl of cAMP-dependent protein kinase (1.25 mg/ml; Sigma Corp.; binding activity 0.09 pmol cAMP/mg) for 60 minutes in an ice-water bath. Preliminary experiments indicated that the binding of [^{3}H]-cAMP reached equilibrium under these conditions. Total [^{3}H]-cAMP binding to the protein kinase was determined as above with the 50 μl of sample replaced with 50 μl of 25 mM TRIS-HCl buffer, pH 7.0. Nonspecific binding of [^{3}H]-cAMP was determined in the presence of 2.5 μM cAMP.

To terminate the incubation after 60 minutes, 70 μl of hydroxyapatite (Sigma Corp.; diluted 1:1, v/v with 10 mM TRIS-HCl buffer, pH 7.0) was added to each sample, the sample vortexed and allowed to stand at least 6 minutes. The samples were then vacuum filtered over Whatman GF/C filters and washed 3 times with 4 ml ice-cold 10 mM TRIS-HCl buffer, pH 7.0. The filters were placed flat in vials and shaken for at least 30 minutes in 1 ml 0.5 N HCl to dissolve the hydroxyapatite. Ten milliliters of Liquiscint scintillation fluid was added to each vial and shaken to form a homogenous mixture. Radioactivity was determined by counting on a Beckman Liquid Scintillation counter. Specific [^{3}H]-cAMP binding was calculated as the difference between total and nonspecific binding. The quantity of cAMP in each sample was determined by standard additions using the percent inhibition of specific [^{3}H]-cAMP binding by each sample. The standard curve was constructed by the same protocol as above using known concentrations of cAMP ranging from 2.5 nM to 500 nM. To confirm the specificity of this assay for cAMP, several of the unknown samples as well as samples with known cAMP content were incubated with 600 μg of 3′,5′-cAMP phosphodiesterase (Sigma Corp.; 0.02-0.05 U/mg) for 80 minutes at 37°C prior to assay. In all cases, preincubation with phosphodiesterase prevented the inhibition of [^{3}H]-cAMP binding to the protein kinase, confirming the specificity of this assay for cAMP.

[^{3}H]-inositol incorporation. Slices were made as described in "Methods," Chapter 2. Slices were washed four times with warm, oxygenated KRB buffer. Following the wash, the slices were allowed to settle and the excess buffer removed leaving enough buffer to comprise

four volumes of settled slices. [^{3}H]-inositol (16.3 Ci/mmol; Amersham Corp.) was added to a final concentration of 0.1 to 0.3 μM. The tube was gassed with O_2/CO_2, capped, and incubated in a shaking water bath at 37°C. Immediately after addition of radioactivity, and at appropriate time points, a 200 μl aliquot of dispersed slices was homogenized with a Tekmar Tissuemizer at setting 50 for 15 seconds. Aliquots were taken for protein determination. A 500 μl aliquot of the homogenate was placed in a Falcon 17 x 100 mm polypropylene tube. Two milliliter of 2:1 methanol:chloroform was added. An additional 0.75 ml of distilled water and 0.75 ml chloroform was added. The tubes were capped tightly and shaken vigorously for 5 minutes then centrifuged for phase separation. The aqueous layer was aspirated and a 200 μl aliquot of the chloroform layer was taken for determination of radioactivity incorporated into membrane lipids. The chloroform was evaporated under a stream of air and OCS scintillation fluid (Amersham) was added. Radioactivity was determined using a Beckman LS7500 liquid scintillation spectrophotometer. The data are expressed as pmols [^{3}H]-inositol incorporated/mg protein/minute.

Results

The effects of acute reserpine treatment on alpha$_1$-stimulated phosphoinositide hydrolysis and beta-stimulated cyclic AMP production. To determine the effects of reserpine treatment on alpha$_1$ stimulated PI hydrolysis, rats were treated (5mg/kg/day) for four days. This regimen depletes brain catacholamines by 90% (Brodie et al., 1966). NE dose-response curves following four days of treatment are shown in Figure 4-1. The ED_{50} values for NE stimulation were 4.17 ± 0.55 μM

for controls and 5.00 ± 1.48 μM for reserpinized rats (n=3). It appears that reserpine treatment does not alter the NE-stimulated PI response.

As a positive control, isoproterenol-induced cyclic AMP production was studied. Reserpine treatment had no effect on basal cAMP production, but increased isoproterenol-stimulated cAMP production from 15.14 ± 1.30 pmol/mg protein to 29.49 ± 1.42 pmol/mg protein ($P<0.001$, n=3). Therefore, reserpine treatment (5mg/kg/day for 4 days) was sufficient to alter beta-adrenergic stimulation of adenylate cyclase but not the $alpha_1$-receptor stimulated PI hydrolysis.

Effect of chronic reserpine treatment on $alpha_1$-stimulated PI hydrolysis and beta-stimulated cyclic AMP production. Since the $alpha_1$-stimulated PI response may require a longer course of treatment before the results of NE depletion are evident, we gave reserpine for 14 days (0.25 mg/kg/day). Dose-response curves are shown in Figure 4-2. The ED_{50} values were 3.26 and 2.71 μM for control and reserpine treated rats, respectively (n=2). Clearly, no increase in $alpha_1$ receptor-stimulated PI hydrolysis is evident with a longer course of reserpine treatment.

Chronic reserpine has no effect on basal cAMP production. Chronic reserpine increased the isoproterenol-stimulated cAMP production from 18.57 ± 1.86 pmol/mg protein to 53.33 ± 4.11 pmol/mg protein ($P<0.001$, n=3). Similar to 4 days of reserpine treatment, 14 days of reserpine are also sufficient to alter beta-adrenergic

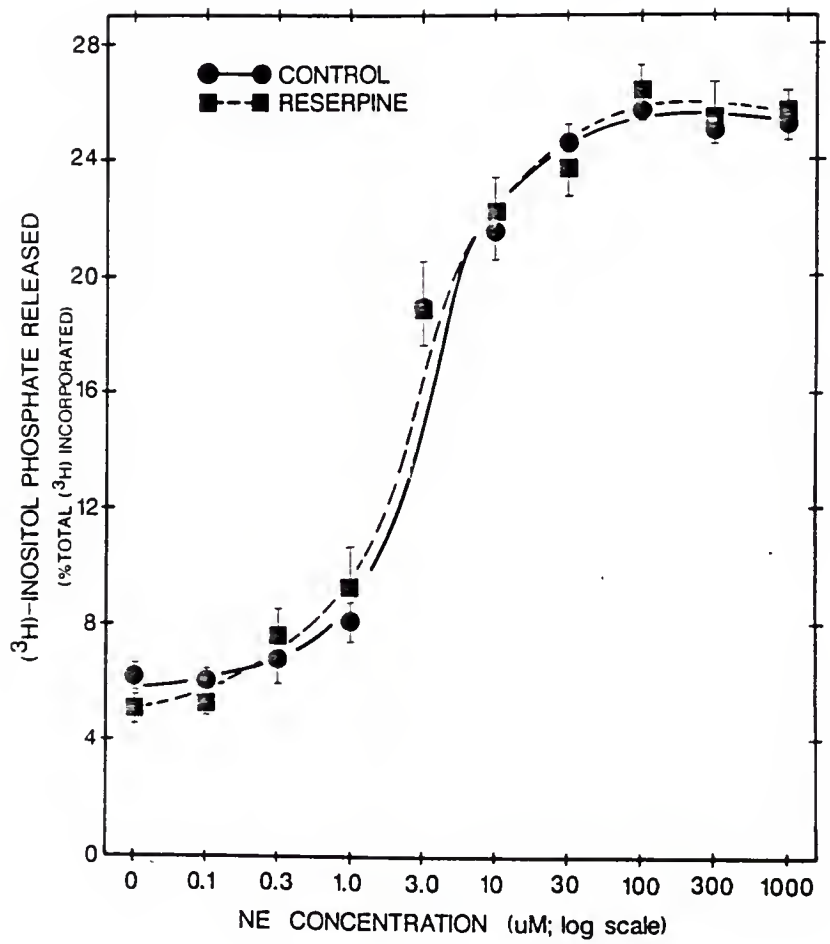


Figure 4-1. Dose-response curves for NE-stimulated PI hydrolysis in rats receiving acute reserpine treatment (5 mg/kg/day for 4 days) vs. vehicle treated. PI hydrolysis was performed as described in "Methods," Chapter 2. Data are expressed as [^{3}H]-inositol released as a percent of total [^{3}H]-inositol incorporated. Each data point represents the average of 9 determinations from 3 separate experiments. Error bars represent S.E.M.

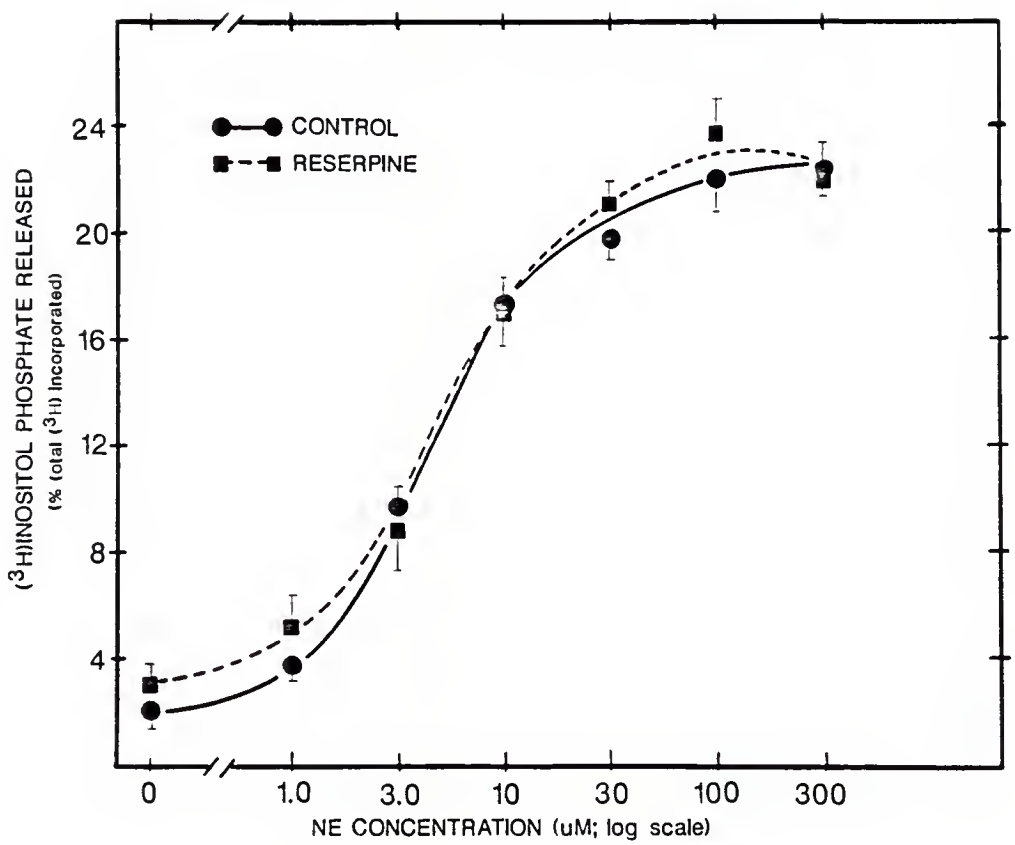


Figure 4-2. Dose-response curves for NE-stimulated PI hydrolysis in rats receiving chronic reserpine treatment (0.25 mg/kg/day for 14 days) vs. vehicle treated. PI hydrolysis was performed as described in "Methods," Chapter 2. Data are expressed as [^{3}H]-inositol released as a percent of total [^{3}H]-inositol incorporated. Each data point represents the average of 6 determinations from 2 separate experiments. Error bars represent S.E.M.

stimulation of adenylate cyclase but not alpha$_1$-stimulated PI hydrolysis.

Effect of chronic reserpine treatment on [^{3}H]-inositol incorporation. To determine if a decrease in [^{3}H]-inositol incorporation was masking the expected increase in the alpha$_1$ stimulated PI responsiveness in reserpinized rats, [^{3}H]-inositol incorporation was examined in rat cortical slices from rats treated with reserpine (0.25 mg/kg/day for 14 days). There was an initial lag phase with rates of 0.015 and 0.014 pmols/mg protein/min for control and reserpine treated, respectively. The secondary rates were 0.0336 and 0.0319 pmols/mg protein/min for control vs. reserpine treated, resepectively (n=2). Thus, no change in inositol incorporation was found.

Discussion

Although many adrenergic systems both in the brain and periphery supersensitize in response to reserpine treatment, we find no increase in alpha$_1$-adrenergic-stimulated PI hydrolysis with 4 or 14 days of reserpine treatment. In contrast, Akhtar and Abdel-Latif (1986) found that denervation of the sympathetic afferents to rabbit iris dilator smooth muscle increased the alpha$_1$-stimulated inositol trisphosphate (TPI) accumulation and the muscle contraction in this tissue. In addition, denervation has no effect on the density of alpha$_1$ receptors from rabbit iris (Page and Neufeld, 1978). These differences may be a result of the model studied. In the cholinergic system, for example, pharmacologic blockade of central cholinergic receptors produces an increase in receptor density but physical lesions of central

cholinergic pathways do not. In addition, the same type of experimental manipulation has different results when comparing central vs. peripheral cholinergic systems (see "Discussion," Chapter 5). Thus, the regulation of $alpha_1$ responses may also be system specific.

Our findings are clearly different than classical descriptions of drug-induced or denervation supersensitivity in the beta-adrenergic system. Reduction of sympathetic stimulation will increase the beta-adrenergic stimulation of adenylate cyclase. Treatment of rats with reserpine will cause a 50 to 100 % increase in NE- and isoproterenol-stimulated adenylate cyclase activity in rat brain (our data, Dismukes and Daly, 1974; Williams and Pirch, 1974). Chemical sympathectomy with 6-hydroxydopamine and chronic denervation also cause an increase in adenylate cyclase activity (Sporn et al., 1976; Weiss and Costa, 1967). Increased beta-adrenergic receptor density was subsequently found to accompany the enhanced cAMP production (Sporn et al., 1976; Weiss et al., 1979; Lefkowitz and Williams, 1978). Thus, reduction of sympathetic input results in supersensitivity due to increased beta-adrenergic receptor-coupled adenylate cyclase activity. The fact that we see no supersensitivity is an unexpected finding. Perhaps rat brain $alpha_1$ receptor-stimulated PI hydrolysis simply does not supersensitize. It is also possible that supersensitivity does occur, but at a mechanism beyond receptor-stimulated PI hydrolysis. We know, for example, in smooth muscle reserpine treatment results in a non-specific supersensitivity to a variety of contractile agents (Nasseri et al., 1985). Changes in resting membrane potential (Fleming and Westfall, 1975), changes in

Ca^{++} permeability or availability (Garrett and Carrier, 1971), and enhanced cell-cell communication (Lee et al., 1975) have all been proposed as possible mechanisms for this non-specific supersensitivity. Thus, it may be that $alpha_1$ receptor-stimulated PI hydrolysis is not an appropriate response to study in this case.

In conclusion, both acute and chronic treatment with reserpine caused an increase in beta-adrenergic receptor-stimulated adenylate cyclase activity, but not $alpha_1$-adrenergic receptor-stimulated PI hydrolysis.

CHAPTER 5
EFFECTS OF nBM LESIONS ON MUSCARINIC-STIMULATION OF PHOSPHOINOSITIDE HYDROLYSIS

Preface

About the same time we were performing the regulation studies with reserpinized rats, Dr. Gary Arendash from the University of South Florida approached us about performing similar regulation studies in rats lesioned at the nucleus basalis magnocellularis. We agreed to perform the experiments and the results of those experiments are in Chapter 4. Although this has little to do with the adrenergic system, it does allow us to compare and contrast the regulation of cholinergic-stimulated PI hydrolysis to the adrenergic system.

Introduction

Clinically Alzheimer's disease presents as a loss of cognitive function (Coyle et al., 1983). This loss of cognitive function is thought to be related to a loss of cholinergic innervation of the cerebral cortex. Patients with Alzheimer's disease have a marked decrease in the number of cholinergic neurons in the nucleus basalis of Meynert (NBM), which projects to the cerebral cortex (Nagai et al., 1983), and a significant loss of cortical choline acetyltransferase (CAT) activity, a marker enzyme for cholinergic nerve terminals. Thus a degeneration of the cholinergic projection from NBM to the cortex occurs in Alzheimer's disease. We have investigated

muscarinic-cholinergic receptors in an animal model of Alzheimer's disease. The nucleus basalis magnocellularis (nBM) in the rat contains cholinergic neurons which project to the cerebral cortex of the rat. Lesions of this nucleus result in decreased performance of rats on memory related tasks and permanent decreases in cortical cholinergic markers (Flicker et al., 1983; Kesner et al., 1986). Thus, chemical lesions of the nBM appear to mimic some of the neurochemical and behavioral changes known to occur in Alzheimer's disease.

Denervation induced receptor supersensitivity is a well known phenomena at certain cholinergic synapses (Burt, 1978; Sachs et al., 1979) and for several other neurotransmitter receptor systems (Fleming et al., 1973). The purpose of this study was to determine the effects of nBM lesions on muscarinic cholinergic receptor recognition sites and on various aspects of phosphoinositide hydrolysis in rat cerebral cortical slices. Muscarinic-cholinergic receptor stimulated phosphoinositide hydrolysis has been well characterized as the second messenger response to post-synaptic muscarinic receptors in rat cerebral cortex (Fisher, et al., 1983; Gonzales and Crews, 1984). We report here that more than three weeks after nBM lesions we find no denervation supersensitivity in cerebral cortical muscarinic receptors. The number of muscarinic-cholinergic receptor recognition sites as determined by [^{3}H]-QNB nor the apparent affinity of the sites for agonists was altered. Furthermore, muscarinic stimulation of phosphoinositide hydrolysis was not changed. These studies suggest that no compensatory increase in muscarinic receptor density and/or

muscarinic responsiveness occurs during the loss of cerebral cortical cholinergic innervation in Alzheimer's disease.

Methods

Animal preparation. Adult male Sprague-Dawley rats, weighing 265 to 335 g at surgery, were used in these studies. Animals were maintained on a 14-h light, 10-h dark schedule and provided with Purina rat chow and water ad libitum. NBM lesions were performed as described previously (Arendash et al., 1972). Animals were anesthetized with sodium pentobarbital (50 mg/kg) and placed in a Trent Wells stereotaxic apparatus. Ibotenic acid was dissolved in phosphate buffered saline (pH 7.4) at a concentration of 5 μg/μl. A 10 μl Hamilton syringe, equipped with a 30 gauge needle, was mounted onto the probe drive of the stereotaxic apparatus and filled with ibotenic acid solution. One microliter of this solution was infused unilaterally into the right nBM at 2 dorsoventral sites (10 μg ibotenic acid total) utilizing the following coordinates: A6.9-7.3, L2.6-2.7, V5.6-5.9 and V6.4-6.7 mm (Konig and Klippel, 1963). Each infusion was done at the rate of 0.5 μl/minute and the needle was left in place for 5 minutes following each infusion. The lesions typically result in an approximately 80 to 90% destruction of the nBM (Arendash et al., 1972). To verify lesions were comparable to those previously characterized several rats were sacrificed, following lesions and frontal cortices assayed for CAT activity according to modifications (Lehman and Fibiger, 1978) of existing methodology (Fonnum, 1969). A 40% decrease in ipsilateral frontal cortex CAT activity was found when cortical CAT activity from the lesioned hemisphere was compared to

activity in the contralateral unlesioned cortex (22.40 ± 3.77 vs. 38.08 ± 0.63 nMole/mg of protein/hr, n = 3). This nBM lesion-induced cortical loss of CAT activity is consistent with a loss of the major extrinsic cholinergic innervation to the neocortex from the nBM.

Phosphatidylinositol hydrolysis. See "Methods" section, Chapter 2.

[^{3}H]-inositol incorporation. Slices were made as described in "Methods," Chapter 2. Slices were washed four times with warm, oxygenated KRB buffer. Following the wash, the slices were allowed to settle and the excess buffer removed leaving enough buffer to comprise four volumes of settled slices. [^{3}H]-inositol (13.8 Ci/mmol; Amersham Corp.) was added to a final concentration of 0.1 to 0.3 μM. The tube was gassed with O_2/CO_2, capped, and incubated in a shaking water bath at 37°C. At appropriate time points, a 50 μl aliquot of packed slices was removed and placed into 800 μl of ice-cold KRB buffer and homogenized with a Tekmar Tissuemizer at setting 50 for 15 seconds. A 500 μl aliquot of the homogenate was placed in a Falcon 17 x 100 mm polypropylene tube. Two ml of 1:2 chloroform/methanol (v/v) was added. An additional 0.75 ml of distilled water and 0.75 ml chloroform was added. The tubes were capped tightly and shaken vigorously for 5 minutes then centrifuged for phase separation. The aqueous layer was aspirated and a 200 μl aliquot of the chloroform layer was taken for determination of radioactivity incorporated into membrane lipids. The chloroform was evaporated under a stream of air and OCS scintillation fluid (Amersham) was added. Radioactivity was

determined using a Beckman LS7500 liquid scintillation spectrophotometer.

[^{3}H]-Quinuclidinyl Benzilate (QNB) Binding. Rats were decapitated and their brains placed in ice-cold 50mM tris(hydroxymethyl)aminomethane (TRIS buffer) containing 1mM EDTA, pH adjusted to 7.4. Tissue from the lesioned and contralateral non-lesioned fronto-parietal cerebral cortex was dissected away and wet weights were determined. The tissues were then homogenized in 20 volumes of ice-cold 50mM TRIS/HCl buffer containing 1mM EDTA pH 7.4 using a Tekmar Tissuemizer at setting 50 for 30 seconds. The homogenate was incubated on ice for 30 minutes. This treatment removes endogenous neurotransmitter from the membranes (Potter et al., 1984). The homogenate was then centrifuged for 10 minutes at 42000 x g at 4°C. The pellet was resuspended in an ice-cold, modified KRB buffer containing: 118 mM NaCl, 4.7 mM KCl, 0.75 mM $CaCl_2$, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 24.8 mM $NaHCO_3$. The membranes were washed twice more with modified KRB buffer and an aliquot was taken for protein determination. Reactions were started with the addition of 120 μg of protein into modified KRB buffer containing 1 nM [^{3}H]-QNB (Amersham) and the appropriate concentration of carbachol in a total volume of 2 ml. Non-specific binding was determined in the presence of 1 μM atropine. Incubations were performed at 37°C for 60 minutes and terminated by rapid filtration onto Whatman GF/B filter paper. Filters were washed 3 times with ice-cold modified KRB buffer. Filters were placed into scintillation vials and 10 ml of Liquiscint

added. Vials were shaken for 1 hour and radioactivity was measured in a Beckman LS7500 scintillation counter.

Data Analysis. Competition curves were analyzed using an iterative, nonlinear curve fitting program as described by McKinney and Coyle (1982) (see "Data Analysis," Chapter 3). ED_{50} values were determined using probit analysis as outlined by Goldstein (1964). The data were analyzed by Student's t-test.

Results

Effect of nBM lesions on muscarinic-stimulated phosphoinositide hydrolysis. To determine the effect of nBM lesions on cortical muscarinic receptor responsiveness we measured carbachol-stimulated hydrolysis of phosphatidylinositol. Dose-response curves for carbachol stimulated phosphoinositide hydrolysis were determined in control contralateral or nBM lesioned ipsilateral frontoparietal cortical slices seven days post-lesion. The dose response curves to carbachol in control and lesioned hemispheres were identical (Figure 5-1). To determine if changes might require longer periods of denervation rats were studied approximately 3 weeks post-lesion. In frontoparietal slices from animals 21 to 23 days post-lesion, again no apparent change in the responsiveness of carbachol-stimulated PI hydrolysis was seen (Figure 5-2). The ED_{50}s were 95 μM ± 20 μM (n = 3), for slices from control hemispheres and 129 μM ± 60 μM (n = 3) in slices from lesioned hemispheres. Although calculated ED_{50} values varied from experiment to experiment, control and lesioned curves were paired within experiments and were consistently similar. Analysis of variance between all carbachol stimulated groups indicates the ED_{50}s

are not significantly different between control and lesioned hemispheres at either one or three week time points (data not shown). Maximal activity was reached at 1 to 3 mM carbachol and was similar for both hemispheres studied.

To determine if nBM lesions alter the incorporation of [^{3}H]-inositol into slices from frontoparietal cerebral cortex, slices were incubated up to one hour and the amounts of [^{3}H]-inositol incorporated into the phosphatidylinositol pool determined. Incorporation was linear to at least 60 minutes and the rate was similar in slices from control and lesioned hemispheres (Table 5-1). These results indicate that ibotenate lesioning produces no change in the ability of cortical slices to incorporate [^{3}H]-inositol 22 days post-lesion.

To investigate the possibility that lesioning can alter the rate of carbachol-stimulated PI hydrolysis, we determined the time course of carbachol-stimulated PI hydrolysis in unilaterally lesioned frontoparietal cortex. In each hemisphere, cortical PI hydrolysis was linear to 15 minutes, and reached plateau levels at approximately 40 minutes (Figure 5-3). At no time point was the hydrolysis in slices from lesioned cortex significantly different from control values.

Since no effect on muscarinic receptor stimulated phosphoinositide hydrolysis was found following unilateral lesions, we compared the PI response to carbachol 25 to 28 days after bilateral ibotenate lesions to that of sham- operated controls. Again, no difference is seen between bilateral lesioned and sham control groups (Table 5-2).

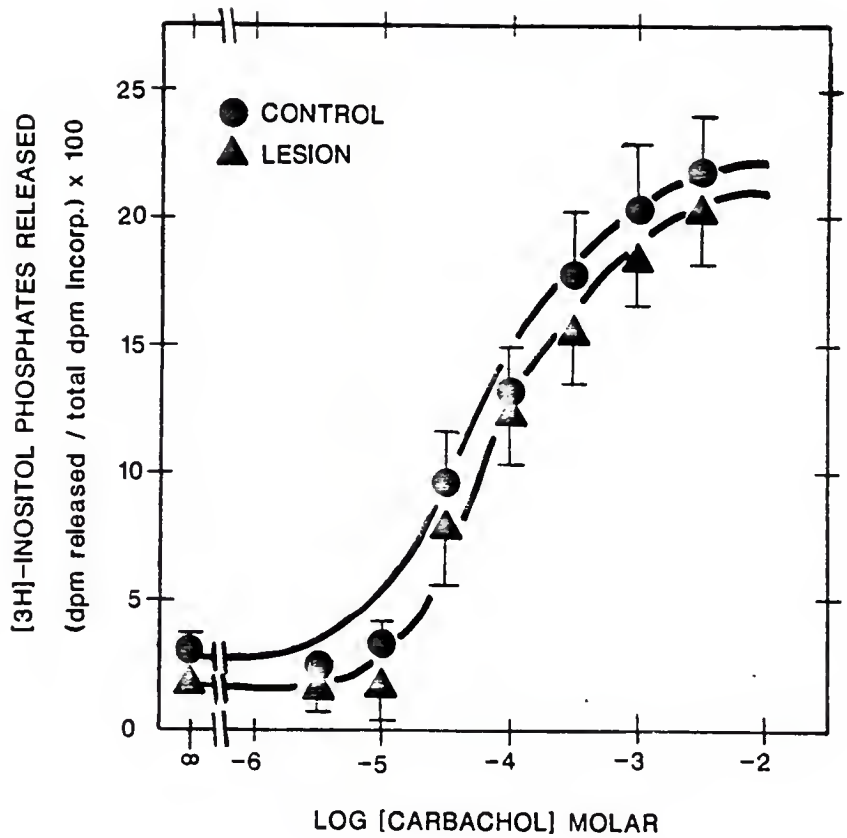


Figure 5-1. Dose-response curves for carbachol-stimulated PI hydrolysis 7 days post nBM lesion. Slices were prepared from the frontoparietal cortex ipsilateral to the nBM lesion. The contralateral frontoparietal cortex served as control. The release of [^{3}H]-inositol phosphates after 60 minutes in the presence of agonist was determined as described in "Methods," Chapter 2. The amount of radioactivity found in the inositol phosphate fraction at time zero was taken as the blank and subtracted from all values. Data points are the means of nine determinations from three separate experiments. Error bars indicate S.E.M. At no carbachol concentration are the two groups significantly different as determined by Student's t test.

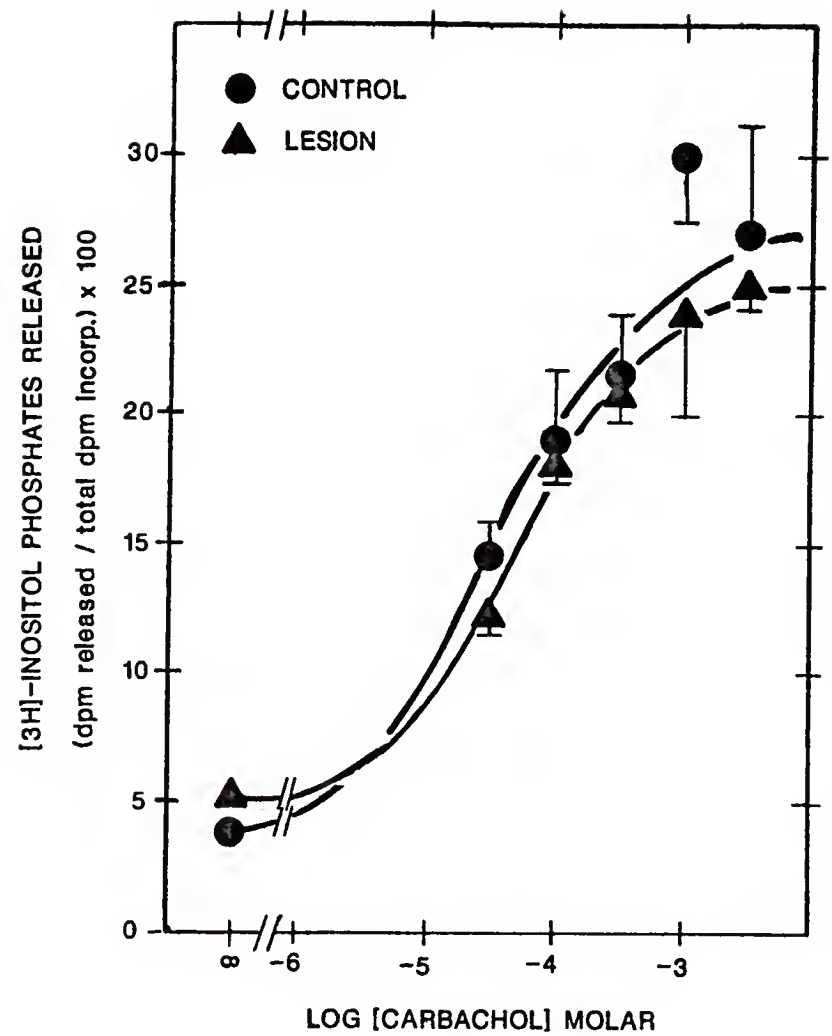


Figure 5-2. Dose-response curves for carbachol-stimulated PI hydrolysis 21 to 23 days post nBM lesion. Data were derived as described in the legend to Figure 4-1. Shown are the means and S.E.M. of at least six determinations from three separate experiments. At no carbachol concentration are the two groups significantly different as determined by Student's t-test.

TABLE 5-1

INCORPORATION OF [^{3}H]-INOSITOL INTO BRAIN SLICES
21-23 DAYS POST NBM LESION

Time (Min)	Control	Lesion
10	21.9 ± 3.3	22.8 ± 3.4
60	165.0 ± 9.9	146.0 ± 10.7
Rate	2.75 pmol/min	2.43 pmol/min

Slices from frontoparietal cortex ipsilateral to nBM lesion were compared to frontoparietal cortical slices contralateral to nBM lesion (control). Slices were incubated with 0.1 to 0.3 μM [^{3}H]-inositol for the indicated time and tested for their ability to incorporate [^{3}H]-inositol (see: "Methods," Chapter 5). Data are expressed as pmol [^{3}H]-inositol incorporated/50 μl packed slices. Means ± SEM represent 4 separate determinations from two experiments.

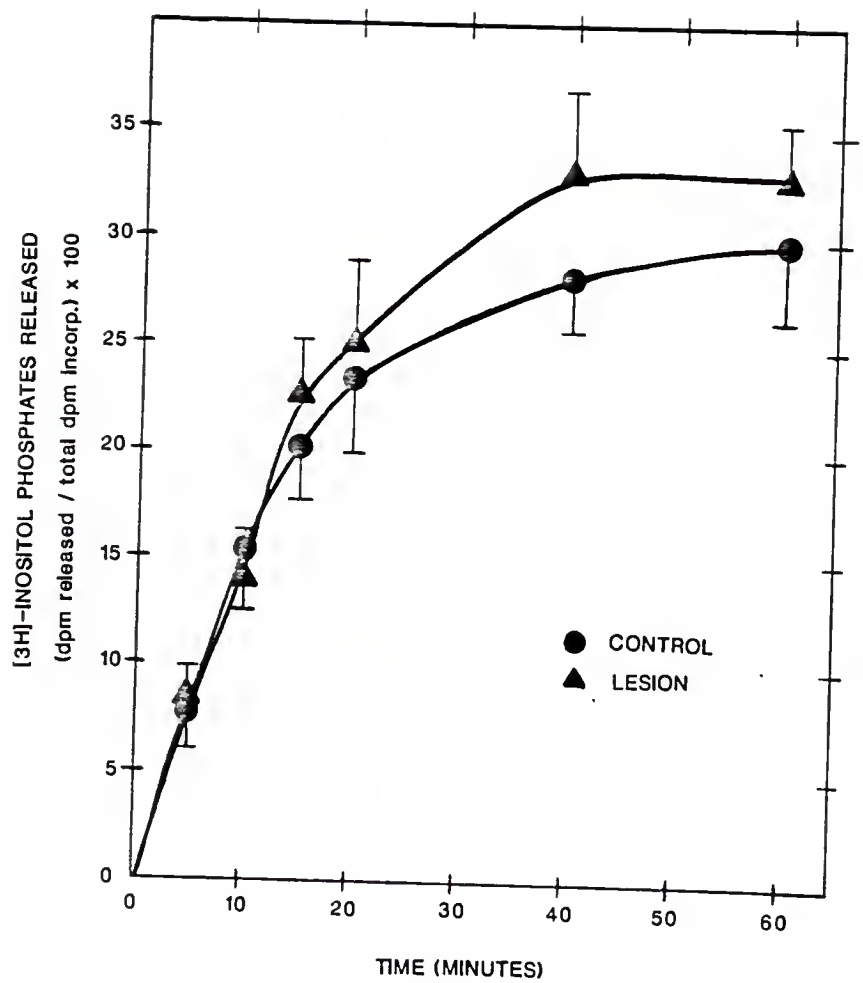


Figure 5-3. Time-course of carbachol-stimulated PI hydrolysis 21 to 23 days post nBM lesion. The release of [^{3}H]-inositol phosphates at various time points was determined as described in "Methods," Chapter 2. Data points represent the mean and S.E.M. of nine determinations from three separate experiments. At no time point was the lesioned data significantly different from controls (t-test).

TABLE 5-2
CARBACHOL-STIMULATED PI HYDROLYSIS IN FRONTOPARIETAL CORTICAL SLICES FROM BILATERALLY LESIONED RATS VS. SHAM-OPERATED CONTROLS

	Control	Lesion
ED_{50} (μM)	26.6 ± 1.0	25.3 (10.3,40.2)
Maximum (10^{-3} M Carb)	31.3 ± 4.2	27.8 ± 1.35

ED_{50} values are expressed as μM. Maximum responses are expressed as DPM [^{3}H]-inositol phosphates released/total DPM [^{3}H]-inositol incorporated x 100. Control maximum values represent nine determinations from three separate experiments. Control ED_{50} value is the average ED_{50} value ± the S.E.M. from three separate experiments. Bilateral lesion maximum values represent six determinations from two separate experiments. Lesion ED_{50} value is the average ED_{50} from two experiments, actual ED_{50} values shown in parentheses.

Effects of nBM lesions on muscarinic receptor density and binding properties. To determine if there were changes in cortical muscarinic binding sites 21 days post nBM lesions [^{3}H]-QNB binding to rat cortical membranes was determined. Scatchard analysis of [^{3}H]-QNB binding (Table 5-3) indicates no change in B_{max} or affinity as determined by K_d values of [^{3}H]-QNB binding to frontoparietal cortical membranes from lesioned and control hemispheres. Again, no difference was found between the two groups. To investigate potential changes in agonist binding, competition curves for [^{3}H]-QNB sites by carbachol were performed. Carbachol exhibited two-site displacement of [^{3}H]-QNB binding as indicated by the gradual slope of the curves in Figure 5-4 which was confirmed by computer modeling (Table 5-4). Membranes from the lesioned hemispheres retained a two-site displacement of [^{3}H]-QNB by carbachol. The distribution of receptors was approximately 30% high affinity and 70% low affinity for carbachol displacement. This distribution was not altered by lesioning. Control K_d values for the high and low affinity sites differed by approximately 100 fold in both control and lesioned hemispheres (Table 5-4). None of the binding parameters were found to be significantly different. Thus, displacement of [^{3}H]-QNB by carbachol is not altered by lesioning.

Effects of nBM lesions on phosphoinositide hydrolysis evoked by other neurotransmitters. To determine if nBM lesions might alter

TABLE 5-3

SCATCHARD ANALYSIS OF [^{3}H]-QNB BINDING IN MEMBRANES PREPARED FROM RAT FRONTOPARIETAL CORTEX 21-23 DAYS POST NBM LESION

	Control	Lesion
K_d (pmol)	513 ± 110	785 ± 302
B_{max} (pmol/mg protein)	3.67 ± 0.42	3.08 ± 0.98
r	0.984	0.991

Membranes were prepared as described in the [^{3}H]-QNB binding section of "Methods." In this case the membranes were incubated with various concentrations of [^{3}H]-QNB and assayed as described. Means ± SEM of nine determinations from three separate experiments are shown from kinetic parameters. Correlations are mean values for three experiments.

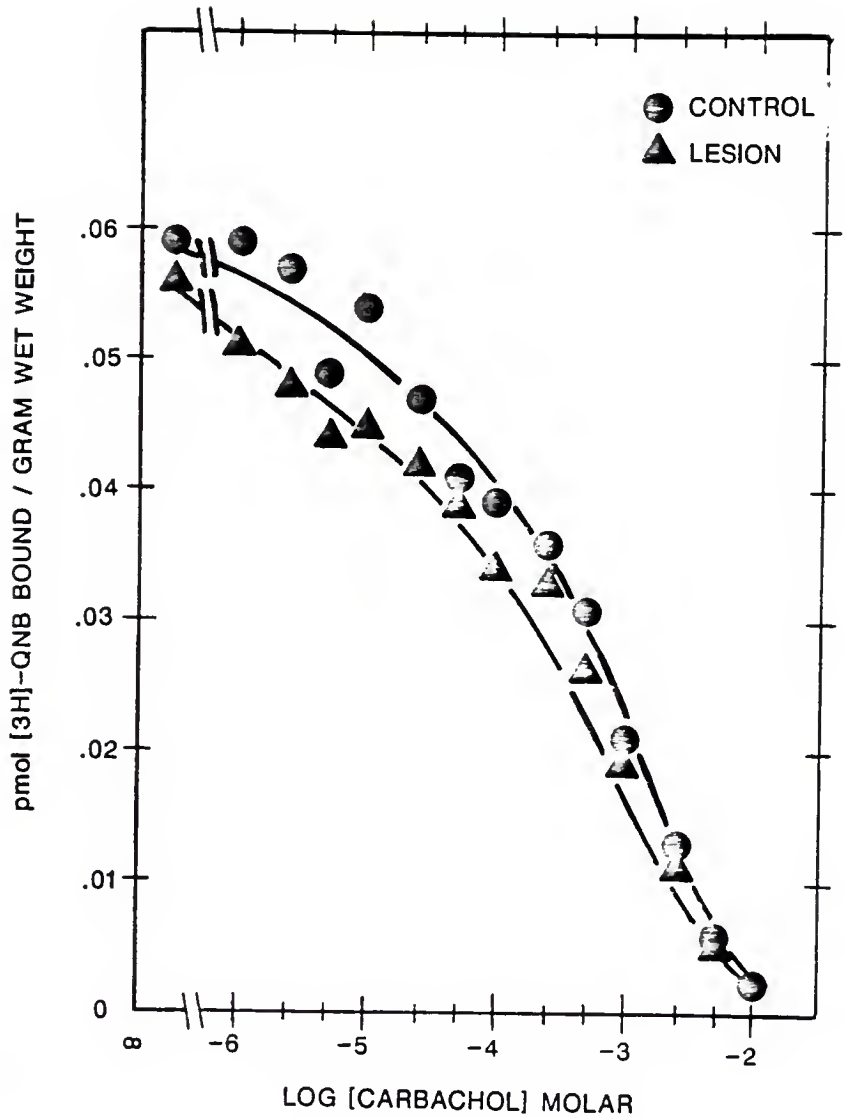


Figure 5-4. Carbachol competition curves for specific [^{3}H]-QNB binding 21 to 23 days post nBM lesion. Membranes were prepared as described ("Methods," Chapter 5) from frontoparietal cortex ipsilateral to the nBM lesion. Membranes prepared from the frontoparietal cortex contralateral to the nBM lesion served as controls. Specific [^{3}H]-QNB binding is expressed as per gram wet weight of tissue. Shown are the means of nine determinations from three separate experiments. The S.E.M. were deleted for the sake of clarity, but at no point were they greater than 10 percent. No significant difference between lesion and control groups exist (t-test).

TABLE 5-4

COMPUTER GENERATED KINETIC PARAMETERS OF CARBACHOL COMPETITION FOR [^{3}H]-QNB BINDING SITES IN RAT FRONTOPARIETAL CORTICAL MEMBRANES 21-23 DAYS POST NBM LESION

	Control	Lesion
K_d high (μM)	1.03 ± 0.55	0.32 ± 0.13
K_d low (μM)	74.95 ± 29.80	57.02 ± 25.55
% high	34.15 ± 4.80	32.43 ± 3.39
% low	65.85 ± 4.80	67.57 ± 3.39

Membranes were prepared as described in the legend to Figure 5-4. Computer analysis is described in McKinney and Coyle (1982). Shown are the carbachol dissociation constants (K_d) for high and low affinity binding and the percentages of high and low affinity binding. Each value is the mean ± S.E.M. of three separate experiments. Competition curves were performed with a carbachol concentration range from 1 μM to 10 mM, with each point done in triplicate.

receptor-stimulated PI hydrolysis by other neurotransmitters the response to other agents was investigated. In both 7 and 21 day post-lesion studies using tissue from unilaterally lesioned rats, the PI hydrolysis dose-response curves for norepinephrine (NE) were identical (Table 5-5). In addition, PI hydrolysis in response to maximal concentrations of the partial muscarinic agonist oxotremorine (100 μM) and the excitatory amino acid glutamate (1 mM) were unchanged by nBM lesions (Table 5-5). Thus, nBM lesions do not appear to alter glutamate, alpha-adrenergic or cholinergic muscarinic receptor stimulated PI hydrolysis.

Discussion

Denervation frequently will produce an increase in the sensitivity of the effector organ (Fleming et al., 1973). The increased sensitivity observed during decreased stimulation is a compensatory homeostatic response to the loss of stimulation. Denervation supersensitivity to acetylcholine has been found in both skeletal muscle (Pestronk and Drachman, 1978) and superior cervical ganglia (Dun et al., 1976). In nBM lesioned rats, we find no evidence for a compensatory increase in muscarinic receptor sensitivity. The number of muscarinic sites was not altered one or three weeks after nBM lesions. This lack of change in receptor number was found with either unilateral or bilateral lesions. These findings are in agreement with similar studies in the cerebral cortex (McKinney and Coyle, 1982), and hippocampus after septo-hippocampal lesions (Ben-Barak and Dudai, 1980a; Yamayura and Snyder, 1974). In addition, it

TABLE 5-5

AGONIST-STIMULATED PI HYDROLYSIS IN BRAIN SLICES
FROM RATS 7 AND 21-23 DAYS AFTER NBM LESION

	Control	Lesion
7 Days Post-Lesion		
Norepinephrine ED_{50} (μM)	5.43	5.31
Maximum	24.10 ± 1.27	25.30 ± 0.93
Carbachol (10^{-3} M)	22.04 ± 1.25	21.50 ± 1.07
Oxotremorine (10^{-4} M)	7.62 ± 1.30	7.41 ± 1.00
Glutamate (10^{-3} M)	12.39 ± 0.74	11.84 ± 0.73
21 Days Post-Lesion		
Norepinephrine ED_{50} (μM)	2.40	5.05
Maximum	23.62 ± 1.29	26.52 ± 0.79
Carbachol (10^{-3} M)	30.00 ± 2.50	24.00 ± 4.50
Oxotremorine (10^{-4} M)	8.92 ± 0.28	10.44 ± 0.67
Glutamate (10^{-3} M)	12.98 ± 0.49	13.30 ± 0.62

Agonist-stimulated PI hydrolysis in brain slices from 7 and 21 to 23 days after nBM lesion. Frontoparietal slices ipsilateral to nBM lesion were compared to the contralateral slices (control). Slices were incubated with agonist for 60 minutes as described in "Methods," Chapter 2. PI hydrolysis is expressed as DPM [^{3}H]-inositol phosphates released/total DPM [^{3}H]-inositol incorporated x 100. ED_{50} values were determined as outlined by Goldstein (1964). Shown is the mean ED_{50} from two experiments. ED_{50} values are expressed as μM. All values are means of six determinations from two separate experiments except for carbachol (9 determinations from 3 experiments.

is consistent with the lack of change in receptor-stimulated PI hydrolysis seen in our experiments. In contrast to these reports, one study which measured muscarinic binding sites only in the dorsal hippocampus found a 20% increase in muscarinic sites after septo-hippocampal lesions (Westlind et al., 1981). Our finding in cerebral cortex suggests that there is no denervation supersensitivity at either the receptor level or the receptor coupling to PI hydrolysis.

Medial septum lesions increase the electrophysiological responsiveness of hippocampal pyramidal cells to acetylcholine but not carbachol (Bird and Aghajanian, 1975). The change in sensitivity to acetylcholine was concluded to result from a loss of pre-synaptic acetylcholinesterase following the lesion, with no change in receptor responsiveness. Thus, this change in sensitivity is not via classical denervation supersensitivity changes in receptor quantity or receptor stimulated second messenger responses.

Although lesions of central cholinergic pathways do not appear to result in denervation supersensitivity, chronic treatment with antagonists will result in the expected increase in receptor density. Chronic atropine (40 mg/kg for at least 5 days) produced an increase in total receptors by 49%, due to a 100% increase in agonist high affinity sites and 25% increase in agonist low affinity sites in the rat cerebral cortex (McKinney and Coyle, 1982). Chronic atropine was found to produce a dose-dependent increase in antagonist binding sites in rat hippocampus (Westland et al., 1981). Treatment of neonatal rats with scopolamine for 20 days resulted in a 20% increase in hippocampal [^{3}H]-QNB binding (Ben-Barak and Dudai, 1980b), whereas

septal lesions in rats 2 to 3 days after birth did not result in an increased number of muscarinic sites in the hippocampus (Ben-Barak and Dudai, 1980a). Inhibition of acetylcholinesterase by organophosphates causes a decrease in muscarinic binding sites in the rat cortex (Costa et al., 1986; McKinney and Coyle, 1982), and other rat brain regions (Gazit et al., 1979), with an associated decrease in the phosphoinositide hydrolysis response to carbachol in the cortex (Costa et al., 1986). Thus, it appears that the central cholinergic system can undergo sensitivity changes in response to pharmacological manipulations.

Although cholinergic receptors increase following denervation in the periphery (Dun et al., 1976; Pestronk and Drachman, 1978), or pharmacologic blockade in the central nervous system, the overwhelming evidence suggests that central lesions of cholinergic pathways do not produce any increase in the number of receptors. It is possible that in the central nervous system, a pre-synaptic factor is required for post-synaptic receptor regulation. In the absence of the pre-synaptic terminals, the factor(s) are unavailable and there is no post-synaptic regulation in response to denervation. Regardless of the mechanism, our data, and that of others suggest that central cholinergic lesions have little or no effect on muscarinic receptor sensitivity (Ben-Barak and Dudai, 1980a; McKinney and Coyle, 1982).

We have studied nBM lesions in the rat as a model for Alzheimer's disease. Our finding that nBM lesions do not induce muscarinic supersensitivity is clouded by the observation that in the rat, lesioning the nBM results in an approximately 50% decrease in

frontoparietal cortical CAT activity (our data; Johnston et al., 1981), whereas, in the brains of some Alzheimer's disease patients larger decreases in cortical CAT activity have been reported (Coyle et al., 1983). Our data indicating no denervation supersensitivity could be due to this residual cholinergic innervation in the rat. However, a variety of behavioral studies in rats indicate that nBM lesions produce memory deficits in rats which mimic the symptomatology of Alzheimer's disease (Flicker et al., 1983; Kesner et al., 1986). Furthermore, the loss of CAT activity in patients with Alzheimer's disease has been correlated with a loss of cognitive function (Perry et al., 1978). An increase in muscarinic receptor sensitivity would be expected to compensate for at least some loss of cholinergic innervation. Taken together these data suggest that the lack of a mechanism for cerebral cortical cholinergic supersensitivity in response to loss of pre-synaptic input may underlie the correlation between the loss of cholinergic innervation and the loss of cognitive function in Alzheimer's disease.

CHAPTER 6
CONCLUSIONS

Norepinephrine and a variety of other adrenergic compounds stimulate PI hydrolysis in the rat brain. Differences in NE-stimulated PI hydrolysis were found when comparing various brain regions. The relative efficacy for partial agonists remained consistent from region to region with the exception of the imidazoline derivative oxymetazoline. In addition, the PI hydrolysis response to oxymetazoline rose sharply from almost zero at 100 μM to maximum at 1 mM. This suggests that oxymetazoline may have a different mechanism of stimulating PI hydrolysis than phenylethylamine derivatives.

Further comparison of imidazolines to phenylethylamines shows that both classes of compounds compete for the tritiated alpha$_1$ antagonist prazosin in a biphasic manner in TRIS buffer. In addition, imidazolines exhibit higher affinities for the [^{3}H]-prazosin site than phenylethylamines. When the competition assays were performed in KRB buffer, the imidazolines retained two-sites of interaction as well as their high affinity, while phenylethylamines were converted to one-site of interaction. Thus, qualitative differences exist between imidazolines and phenylethylamines with respect to alpha$_1$ receptor interactions.

The ability of phenylethylamines and imidazolines to stimulate PI hydrolysis was examined. Phenylethylamines exhibit dose-respose

relationships with respect to PI hydrolysis, with norepinephrine, epinephrine, and alphamethylnorepinephrine being full agonists. Partial agonists include 6-flouronorepinephrine, phenylephrine, and methoxamine. Imidazoline-stimulated PI hydrolysis, although dose-related, does not exhibit classical dose-response characteristics. This provides further evidence that imidazolines stimulate PI hydrolysis via an alternative mechanism.

Comparison of K_d values from competition curves and ED_{50} values obtained from agonist-induced PI hydrolysis demonstrate a strong correlation between K_d values obtained in KRB buffer and ED_{50} values for phenylethylamines but not imidazolines. Correlation coefficients fall off sharply when the K_d values obtained using TRIS buffer are compared to ED_{50} values. Thus, it is likely that the PI hydrolysis observed for phenylethylamines is a result of interaction of these compounds with the $alpha_1$ receptor, whereas the imidazoline-induced PI hydrolysis is not due to their interaction with the $alpha_1$ receptor. In additon, the data also suggest that physiologic buffers may be more appropriate than TRIS buffer for studying $alpha_1$ binding characteristics.

The $alpha_1$ receptor antagonist prazosin was able to inhibit the PI response to all phenylethylamines tested. In contrast, prazosin was unable to inhibit imidazoline-induced PI hydrolysis. In fact, a wide variety of neurotransmitter receptor antagonists were unable to inhibit imidazoline-induced PI hydrolysis. These results provide the final proof that phenylethylamines evoke PI hydrolysis via interaction with the $alpha_1$ receptor, and imidazoline-stimulated PI hydrolysis is

unrelated to their interaction with the $alpha_1$ receptor. In addition, imidazolines exhibit a dose-dependent inhibition of NE-stimulated PI hydrolysis and, therefore, can be classified as $alpha_1$ receptor antagonists. Furthermore, the inability of other antagonists to inhibit imidazoline-induced PI hydrolysis suggests a previously uncharacterized imidazoline receptor or a non-receptor mediated mechanism to account for imidazoline-induced PI hydrolysis.

Investigations into the regulation of $alpha_1$ receptor-stimulated PI hydrolysis in the rat cerebral cortex reveals that both acute and chronic treatments with reserpine do not cause an increase NE-stimulated PI hydrolysis. In contrast, both acute and chronic reserpine treatments produced an increase in beta-adrenergic receptor-stimulated adenylate cyclase activity. Thus, it appears that reduction of NE levels in the brain caused by reserpine treatment is not sufficient to bring about an increase in $alpha_1$ receptor stimulated PI hydrolysis. Clearly, the regulation of $alpha_1$ receptor-stimulated PI hydrolysis differs from the regulation of the beta-adrenergic system.

Reduction of the cholinergic input to the frontoparietal cerebral cortex by lesioning the nucleus basalis magnocellularis does not result in increased carbachol-stimulated PI hydrolysis. Nor does lesioning produce any change in parameters that are likely to mask an increased response such as [^{3}H]-inositol incorporation, the rate of carbachol-stimulated PI hydrolysis, and agonist binding to muscarinic receptors. Like NE-stimulated PI hydrolysis, carbachol-stimulated PI hydrolysis is not affected by loss of neurotransmitter input.

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BIOGRAPHICAL SKETCH

Robert Emil Raulli was born on September 28, 1957, in the sprawling metropolis of New York City, where he grew to be a wise-guy juvenile delinquent so typical of that city. He graduated from John Adams High School and went on to York College of the City University of New York to study chemistry. He later transferred to Arnold and Marie Schwartz College of Pharmacy of Long Island University and graduated with a B.S. in pharmacy in 1981. Realizing, even before he graduated, that he would be bored practicing pharmacy, he enrolled at Albany Medical College to study toxicology in 1981. After two years of dissatisfaction, rumors of the demise of the Toxicology Department, and arctic winters, he transferred to the University of Florida to study pharmacology. Under the supervision of Dr. Fulton Crews, he was awarded his Ph.D. in 1987.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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